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Studies Of The Pharmacological Effects Of Benzoic Acid Analogs On Thrombosis And Inflammation

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STUDIES OF THE PHARMACOLOGICAL EFFECTS OF BENZOIC ACID ANALOGS ON THROMBOSIS AND INFLAMMATION

by

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Abstract

The use of ASA (2-acetoxybenzoic acid) as an anti-inflammatory agent has been hampered by its antiplatelet effects. ASA has recently been the subject of intensive clinical investigation in an effort to determine if these platelet effects result in a reduced incidence of various thrombosis-related events including myocardial and cerebral infarction. A beneficial effect of ASA however, was evident in only some conditions.

Previous work in this laboratory demonstrated that a series of benzoic acid analogs, with only minor structural variation compared to ASA, displayed a wide variation in biological effects compared to ASA. The compounds studied include 2-propionyloxybenzoic acid (2-PBA), 3-propionyloxybenzoic acid (3-PBA), 2-acetylbenzoic acid (ABA), 3-methylphthalide (3-MP) and 3-hydroperoxy-3-methylphthalide (3HMP). The purpose of this study was to extend our knowledge of the effects of these compounds and the processes they modify by assessing the in vivo antithrombotic and anti-inflammatory activities and the inhibition of prostaglandin (PG) synthesis (including PGI_2) in related in vitro systems. The ultimate aim of this work is the design of agents with more specific anti-inflammatory and antithrombotic activities.

The systems studied were 1) ADP and collagen-stimulated platelet aggregation and release of ATP 2) platelet PG synthesis products 3) platelet cyclic nucleotide phosphodiesterase activity (PDE) 4) aorta PGI_2-like activity production 5) aorta arachidonic acid (AA) metabolism 6) electrical injury-induced thrombosis in a rat model

7) carrageenin-induced pleurisy in a rat model and 8) polymorphonuclear neutrophil (PMN) AA metabolism.

No agent inhibited platelet function without interfering with blood vessel PGI_2-activity as measured by bioassay. This demonstrates the similarity in the control mechanisms of platelets and vascular cells. 3-MP inhibited PGI_2-activity but not directly through cyclo-oxygenase inhibition. 3-MP did inhibit platelet PDE activity and it is proposed that the effect of 3-MP on platelets and on PGI_2-activity is through PDE inhibition.

ASA, 2-PBA and ABA were the major inhibitors of *in vivo* thrombus formation. These agents appeared however, to exert their effects through mechanisms not directly related to the irreversible inhibition of platelet function. ABA, in fact, did not inhibit platelet PG synthesis.

PMNs, under stimulated or nonstimulated conditions did not convert ^{14}C-AA to PGs, however, there was an interaction between platelets and PMNs in the formation of AA metabolites. Serum treated zymosan (STZ)-stimulated PMNs did (i) metabolize AA to unique products which co-chromatographed with PGs and (ii) incorporate large amounts of AA into membrane phospholipids. These processes were completely abolished by 3HMP while 3-MP caused alterations in the profile of products.

In the rat pleurisy model, 3HMP caused profound inhibition of PMN accumulation suggesting a role for PMN AA metabolites. 3-MP was also a potent inhibitor of PMN accumulation, likely working through a PDE-related mechanism. 3-PBA, an agent found to potentiate rather than inhibit PG-mediated events, caused a significant increase in exudate volume but, at the same time, reduced PMN accumulation. These data provide evidence that exudate formation is increased by PGs which block

PMN accumulation. These data also suggest a dissociation between PMN accumulation and exudate formation in inflammation.

ABA appears to be a unique agent with both anti-inflammatory and antithrombotic activity not accountable by PG synthesis or PDE inhibition. This agent appears a likely candidate as an anti-inflammatory agent without antiplatelet activity. The hydroperoxide, 3HMP, appears as a particularly useful tool for the study of the mechanisms of cyclo-oxygenation and lipoxygenation of AA and the activation and inhibition of the enzyme systems involved.

These studies point to unique mechanisms of action of antithrombotic and anti-inflammatory activities with a series of benzoic acid analogs and suggest that PG synthesis inhibition does not totally account for either antithrombotic or anti-inflammatory activity.

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"Whoever labors to penetrate the secrets of reality with a humble and steady mind is being led by the hand of God, even if he remains unaware of it."

-Text of the constitution of the Second Vatican Council as quoted by Pope John Paul II in his address at the Einstein session of the Pontifical Academy of Sciences, Vatican City, 10 November, 1979.

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GLOSSARY

A23187:	divalent cation ionophore A23187
AA:	arachidonic acid
ABA:	2-acetylbenzoic acid
ADP:	adenosine diphosphate
ASA:	2-acetoxybenzoic acid (aspirin)
ATP:	adenosine triphosphate
C3b:	complement component 3b
C5b:	complement component 5b
cAMP:	adenosine-3',5'-cyclic monophosphate
cGMP:	guanosine-3',5'-cyclic monophosphate
Ci:	Curie - measure of radioactivity = 2×10^{12} disintegrations per minute
20-COOH-LTB_4:	carboxy metabolite of LTB_4
CP/CPK:	creatine phosphate/creatine phosphokinase; converts ADP to ATP
CPM:	counts per minute, measure of radioactivity
DMSO:	dimethylsulfoxide
DPM:	disintegrations per minute, measure of radioactivity
ESR:	external standards ratio, method for determining radioactivity counting efficiency
ETYA:	eicosatetraynoic acid; analog of AA but with triple instead of double bonds. Blocks cyclo-oxygenase and lipoxygenase activity.
FMLP:	formyl-methionyl-leucyl-phenylalanine; chemotactic peptide

HETE:	hydroxyeicosatetraenoic acid; lipoxygenase product of AA metabolism
HHD:	hydroxyheptadecadienoic acid
HHT:	hydroxyheptadecatrienoic acid
3HMP:	3-hydroperoxy-3-methylphthalide
H_2O_2:	hydrogen peroxide
HPETE:	hydroperoxyeicosatetraenoic acid
5HT:	5-hydroxytryptamine
ID_{50}:	concentration of drug causing 50% inhibition of an effect
IR:	infrared spectroscopy
IT:	% increase in time to thrombosis
6-keto-$PGF_{1\alpha}$:	stable breakdown product of PGI_2
LTB_4,C_4,D_4 etc:	leukotrienes
MeOH/$CHCl_3$:	methanol/chloroform mixture
MIX:	1-methyl-3-isobutylxanthine
3-MP:	3-methylphthalide
NDGA:	nordihydroguaiaretic acid
NMR:	nuclear magnetic resonance spectroscopy
NSAID:	nonsteroidal anti-inflammatory drug
$O_2^{\cdot -}$:	superoxide anion
20-OH-LTB_4:	hydroxy metabolite of LTB_4
PAF:	platelet activating factor
2-PBA:	2-propionyloxybenzoic acid
3-PBA:	3-propionyloxybenzoic acid
PDE:	cyclic nucleotide phosphodiesterase
PG:	prostaglandin

PDGF:	platelet derived growth factor
PGI_2:	prostacyclin
PGI_2Na^+:	prostacyclin sodium salt
PMN:	polymorphonuclear neutrophilic leukocyte
POPOP:	P= phenyl O= oxazole -secondary fluor in liquid scintillation counting
PPO:	P= phenyl O= oxazole -primary fluor in liquid scintillation counting
RCS:	rabbit aorta contracting substance (contains thromboxane and endoperoxides)
Solvent I:	acetic acid/ethyl acetate (1:99, v:v)
Solvent II:	ethyl acetate/acetic acid/isooctane/water (110:20:50:100, v:v)
Solvent III:	benzene/dioxane/acetic acid (20:10:1, v:v)
SRS-A:	slow reacting substance of anaphylaxis (may contain leukotrienes)
STZ:	serum-treated zymosan
TIA:	transient ischemic attacks
TLC:	thin layer chromatography
TXA_2:	thromboxane A_2
U.V.:	ultraviolet light
VK774:	dipyridamole analog

Chapter 1 GENERAL INTRODUCTION TO THE THROMBOTIC AND INFLAMMATORY PROCESSES AND THE USE OF SALICYLATES

Thrombosis

Thrombosis is the abnormal formation of a solid mass of constituents of the blood in the living blood vessel or heart. Thrombi arising in the venous flow resemble clotted blood *in vitro*, while thrombi arising in the arterial or cardiac circulation (rapid moving) are composed largely of fibrin and platelets (Robbins and Cotran 1979).

Three major factors leading to thrombosis are 1. injury to the endothelium 2. alterations in normal blood flow 3. alterations in the blood.

The process of thrombogenesis may be briefly summarized as follows. Injury to the endothelial cell layer which lines the blood vessels releases tissue factors and exposes subendothelial connective tissues. Platelets adhere and the plasma clotting system is triggered. Platelets degranulate and prostaglandin (PG) production occurs. Platelet aggregation caused by released adenosine diphosphate (ADP) and vasoconstriction caused by released serotonin (5HT) and thromboxane A_2 (TXA_2) result in a primary (temporary) hemostatic plug. Thrombin, TXA_2 and PG endoperoxides promote the release reaction and irreversible aggregation. The amorphous platelet mass and trapped red cells are enmeshed in fibrin to form a definitive (permanent) hemostatic plug. Endothelial plasminogen activator and plasma antithrombin check rapid clotting. Clot retraction (platelet contraction) and fibrinolysis reduce

the size of the plug. Organization (ingrowth of capillaries and fibroblasts, infiltration by polymorphonuclear leukocytes and macrophages) and endothelial regeneration gradually repairs the injured area (Gimbrone 1979). The importance of each individual factor to the development of thrombosis is a matter of discussion and study (Mustard and Packham 1981).

Endothelial injury can occur due to a variety of causes. In atherosclerosis, ulcerated plaques formed in the artery are sites subject to thrombus formation despite rapid blood flow (Ross and Harker 1976). Damage to endothelium in heart chambers results from myocardial infarction, infection and immunological reactions. Hemodynamic stress in hypertension and chemicals from a variety of sources such as cigarette smoke, bacterial toxins and those of endogenous origins such as homocysteine, which is due to an inborn error of metabolism, can cause denudation of the endothelium and subsequent thrombosis. In the leg veins, areas of varicosity and incompetence of venous valves result in sluggish venous drainage, and here thrombosis is common (Robbins and Cotran 1979). Changes in blood flow and hypercoagulability are discussed by Robbins and Cotran (1979).

Salicylates in the Treatment of Thrombosis

Ingestion of acetylsalicylic acid (ASA) by normal individuals causes a prolongation of the bleeding time that is not due to hypoprothrombinemia. A dose of 0.325-1.3 g of ASA will prolong the bleeding time of normal patients for a period of 4-7 days after the drug is discontinued (Weiss 1978) and this occurs despite the fact that the ASA is cleared from the body within hours. ASA prolongs the bleeding

time by inhibiting various platelet functions, probably through inhibiting the enzymes responsible for PG production (Smith and Willis 1971).

ASA has been the subject of a number of long range studies in various disease processes in which platelets and thrombosis have been implicated (Circulation 1980).

A number of studies have tested the effects of ASA and other agents on myocardial infarction using mortality as the end point. Of these trials, the Aspirin Myocardial Infarction Study (AMIS, 1000 mg/day), Elwood II (900 mg), Persantin-Aspirin Reinfarction Study (PARIS, 972 mg) and the Aspirin Reinfarction Trial (ART, 800 mg) all had sufficient numbers of patients to make the trial statistically valid. In all these studies, except AMIS, there was a reduction in mortality in the ASA treatment groups, however the results were not statistically significant. In the AMIS trial there was actually a slight increase in mortality with ASA. The majority of all patients studied belonged to either the AMIS or PARIS studies and from these studies it was concluded that ASA had no beneficial effect on mortality in patients who started ASA therapy several months after a myocardial infarction. The effects of ASA on those entering into therapy after a shorter time period after the first infarction are still not conclusive and require further clinical investigation.

Two major trials of ASA in cerebral ischemia include Aspirin in Transient Ischemia (AITIA) and Recent Recurrent Presumed Cerebral Emboli (RRPCE), both of which used a dose of 1300 mg ASA/day. In these studies there was a substantial reduction in transient ischemic attacks (TIA), stroke and death especially from vascular causes. In AITIA there was a

31% reduction in TIA with ASA alone and this was highly significant. The effects were predominantly in males however (Genton 1980).

ASA has proven useful in reducing thrombosis associated with heart valve prosthesis, ischemia associated with thrombocytopenia and in arteriovenous shunts. It was not useful in aorta coronary bypass grafts and the use is questioned in venous thrombosis.

Because of problems in some clinical trials concerning the number of patients and inconclusive data (Meier 1981, Peto and Yusuf 1981), the clinical use of ASA in thrombosis has not gained widespread acceptance. Further clinical trials, examining the effects of ASA on morbidity and mortality in cerebrovascular disease, coronary heart disease and diabetic retinopathy, are still in progress (Passamani 1980).

Inflammation

Acute inflammatory reactions usually follow a basic pattern although the duration and intensity of the reaction depends on the severity of the injurious agent and the reactive capablity of the host. Also, depending on the severity of the injury and the adequacy of the host defence system, the inflammation may remain localized to its site of origin or may evoke a systemic response (Robbins and Cotran 1979).

The usual signs of inflammation are heat, swelling, redness, pain and loss of function (Cohnheim 1889). Local heat and redness result from dilation of the microcirculation in the injured area. The swelling results from the escape of cells, plasma protein and fluid from the blood into the perivascular areas. The mechanism of pain in the inflammatory response is unclear, although PGs, together with bradykinin or 5HT or increased tissue tension due to edema, can induce overt pain

(Robbins and Cotran 1979). The acute inflammatory response may be accompanied by exudate, transudate and pus. Exudate is an inflammatory extravascular fluid that has a high protein concentration and much cellular debris. Transudate is a fluid with a low protein concentration which is an ultrafiltrate of blood plasma and which results from hydrostatic alterations across the vascular endothelium. Pus is an inflammatory exudate containing numerous leukocytes, especially polymorphonuclear leukocytes and parenchymal cell debris. The viscosity of this material depends on the proteolytic activity of lysosomal enzymes present here (Florey 1970, Miller et al 1978).

The major components of the acute inflammatory response include changes in vascular flow, changes in vascular permeability and leukocyte exudation.

Persistant inflammatory stimuli lead to chronic inflammation (Robbins and Cotran 1979). Chronic inflammatory responses are marked by infiltration by mononuclear cells (macrophages, lymphocytes and plasma cells), proliferation of fibroblasts and proliferation of small blood vessels. Chronic inflammation may follow acute inflammation in the case of a persistent stimulus, may follow repeated bouts of acute inflammation or may begin as a low grade smouldering response that never shows signs of acute inflammation. Although polymorphonuclear leukocytes (PMNs) are considered hallmarks of acute inflammation, some forms of chronic inflammation, lasting for months such as osteomyelitis and actinomycosis, continue to show large numbers of PMNs and pus.

Salicylates in the Treatment of Inflammation

Salicylates have been used for many years for their antipyretic

properties, for analgesia against pain of low to moderate intensity especially when associated with inflammation and, especially, for anti-inflammatory activity in the treatment of musculoskeletal disorders such as rheumatoid and osteoarthritis and ankylosing spondylitis. There is a reasonably good rank order correlation between the inhibition of PG cyclo-oxygenase and the anti-inflammatory potency of various ASA-like drugs as estimated in the carrageenin-induced edema test in the rat hind paw (Ham et al 1972). This would suggest that at least part of the anti-inflammatory activity of these agents is mediated through inhibition of PG synthesis. In the treatment of rheumatoid arthritis, salicylates are the first line of therapy and are used for their beneficial anti-inflammatory and analgesic effects (Miller et al 1978). Salicylates are enough to control the majority of patients. This class of drugs can be shown objectively to produce measurable anti-inflammatory changes when given in large doses for long periods to patients with rheumatoid disease. In acute rheumatic fever, salicylates provide relief of pain, swelling, immobility, local heat and redness of the involved joints. Fever and pulse rate are lowered. Severe carditis and heart failure may be reduced by salicylate medication. Other aspects of the disease including chorea, encephalopathy, subcutaneous nodules and other features are not prevented or improved. The progression of this disease and the later phases of granulomatous inflammation and scar formation are not altered with salicylates (Flower et al 1980).

Other actions of salicylates that may contribute to their anti-inflammatory activity include uncoupling oxidative phosphorylation and inhibition of cellular respiration, interaction with cellular proteins and kallikrein, stabilization of lysosomal membranes,

inhibition of the synthesis of mucopolysaccharides, inhibition of histamine release, inhibition of 5-hydroxytryptophan decarboxylase and suppression of antibody production and antigen-antibody reaction (Miller et al 1978).

Objectives

The overlap of antiplatelet and anti-inflammatory activities have hampered the use of ASA and other nonsteroidal anti-inflammatory drugs (NSAID) in inflammatory diseases. At the same time, based on clinical studies, the antithrombotic action of ASA appears complex and requires further definition. This work involves the study of a series of agents with only minor structural differences from ASA but which have been shown to cause a wide spectrum of pharmacological effects in in vitro and in vivo systems related to thrombosis and inflammation. The major focus concerns the correlation of antithrombotic and anti-inflammatory actions of these agents as determined using animal models, with the effects on PG production in related in vitro systems. Inhibition of PG synthesis is presently considered the fundamental mechanism of action of ASA. Where correlations with the inhibition of PG synthesis do not occur, other possible sites of action are considered. By increasing our knowledge of the activities of this group of agents, we propose to come to a clearer understanding of the mechanisms of action of PGs and ASA in these pathological processes and to uncover other, as yet unknown, mechanisms of action by which ASA exerts its effects.

The specific questions addressed at the onset of this project involved the benzoic acid analogs 2-propionyloxybenzoic acid (2-PBA), 3-propionyloxybenzoic acid (3-PBA), 2-acetylbenzoic acid (ABA),

3-methylphthalide (3-MP), 3-hydroperoxy-3-methylphthalide (3HMP) and ASA (2-acetoxybenzoic acid).

In the studies of ASA in the control of thrombosis and inflammation, the following questions were addressed here:

1. In light of the fact that some of these analogs of ASA inhibit platelet aggregation without inhibiting platelet PG synthesis (3-MP, Cerskus 1978) and that 3-PBA appears to augment platelet aggregation and the production of proaggregatory substances by platelet microsomes (Mills et al 1974, Cerskus 1978) is it possible to separate the effects of these agents on collagen-induced aggregation and ADP-induced second phase aggregation from the effects on the simultaneous release reaction? This would provide further information concerning the central role of PG production in platelet aggregation and its relation to the release reaction.

2. As inhibition of total PG synthesis does not explain the inhibition of platelet aggregation by all agents examined, and certain PG synthesis inhibitors do not display typical nonsteroidal anti-inflammatory drug (NSAID) effects in platelet aggregation studies (3HMP), do these agents inhibit platelet aggregation through other likely mechanisms such as inhibition of TXA_2 synthesis or elevation of cAMP through the inhibition of platelet PDE?

3. Is it possible to separate antiplatelet PG and/or antiplatelet aggregation activity from the effects on PGI_2 activity produced by aorta rings using these analogs? If the balance of proaggregatory PG

endoperoxides and antiaggregatory PGI_2 is an important regulator of thrombosis, agents with these characteristics would be likely candidates as antithrombotics.

4. Do these agents inhibit thrombus formation in an in vivo rat model of thrombosis? If so, can the effects be explained by any of the known mechanisms of action of ASA or any of the above information or, do other mechanisms of action appear to be operating?

5. In studies of the benzoic acid analogs in the rat paw carrageenin-induced edema model, agents such as ASA and 2-PBA, which inhibit platelet PG synthesis, displayed anti-inflammatory activity however 3-MP and ABA, which were weak platelet PG synthesis inhibitors, also had anti-inflammatory activity. 3-PBA actually increased inflammation in this model. Can the effects of these agents be reflected in the effects on PG synthesis from another tissue source related to inflammation such as polymorphonuclear neutrophilic leukocytes (PMNs) which have been reported to produce PGs or is the anti-inflammatory effect mediated through another mechanism not related to PGs?

6. Can the effects of these agents on inflammation be reflected in effects on PMN accumulation in vivo in the rat pleurisy model? This would increase our understanding of mechanisms of anti-inflammatory activity by implicating the PMN, a cell commonly found at sites of inflammation, in the mechanism of anti-inflammatory activity of NSAID.

7. Based on the report that ABA has anti-inflammatory activity but does not inhibit PG synthesis (Cerskus 1978), is this agent a possible alternative to ASA in that it is not associated with the inhibition of platelet function?

Chapter 2 THROMBOSIS AND ASA

2.1 Historical Notes, Current Concepts

The Platelet: Historical Notes

The interaction of blood with blood vessels was described by Joseph Lister in 1857 when he reported that blood, in contact with normal blood vessels, remained fluid while that in inflamed vessels took on a different consistency and colour (Lister 1857). Lister confirmed that this was a characteristic of an interaction between the two components as blood samples, removed from normal and inflamed vessels, were identical.

The platelet was identified as early as 1842, however, it was not described as a distinct morphological entity until the work of Bizzozero in 1882. Bizzozero recorded the growth and embolization of thrombi in mildly injured small arterioles and, although his work closely followed that of Wharton-Jones (1851), Bizzozero emphasized the platelet and its central role in the blood coagulation process. The evolution of early knowledge of the platelet and the thrombotic process was thoroughly reviewed by Tocantins (1948).

The role of the platelet in the hemostatic process was further elucidated by Duke (1912) who demonstrated the technique of bleeding time measurement and showed an inverse relationship between bleeding time and platelet count (Poole and French 1961). H.P. Wright (1941) used platelet adhesion to glass as a measure of platelet function. Chandler

(1958) developed a device in which native blood flowed in rotating loops of plastic tubing and thrombi formed due to the generation of thrombin. These techniques allowed the first measurements of platelet function *in vitro*.

The function of the red cells on the adhesion of platelets to glass (Hellem 1960), the importance of adenosine diphosphate (ADP) in this process (Born 1962) and ultimately the development of the Born aggregometer in the early 1960's changed the direction of thrombosis research. With the rapid increase in knowledge of platelet function that followed, the platelet became a model for many other biochemical and physiological phenomena. The platelet has also served as a stepping stone leading to such important discoveries in the thrombosis field as the identification of the presence and biological properties of TXA_2, other PGs and prostacyclin (PGI_2) (Moncada *et al* 1976).

The Blood Vessel: Description

Nineteenth century scientists recognized that along with blood constituents and changes in the pattern of blood flow, the vessel wall was also important in influencing the genesis of thrombosis.

All blood vessels are lined by a single cell layer called the endothelium, which is supported by a basement membrane and these two structures, together with a small amount of loose mesh connective tissue constitute the tunica intima. The intima may contain a few, longitudinally oriented, myointimal cells which are morphologically similar to smooth muscle cells. Intimal proliferation of smooth muscle cells, as occurs in fibromuscular plaque, may be considered a form of atherosclerosis (Ross and Harker 1976).

Vascular endothelium performs many synthetic and metabolic functions. Endothelial cells produce PGs, factor VIII, basement membrane, plasminogen activator and heparin-like anticoagulant substances. Evidence exists that these cells possess receptors for such powerful vasoactive agents as angiotensin II and hormones such as insulin and endothelial cells can actively contract (Robbins and Cotran 1979). The role of the vascular intima in thrombosis has been reviewed by Stemerman (1974). The endothelia, if left undisturbed, appear to be remarkably stable cells having an extremely low turnover rate (Algire and Chalkley 1945). Although controversy exists as to whether platelets adhere to damaged endothelium (Baumgartner 1972), thrombin-stimulated platelets have been shown to adhere to cultured endothelial cells (Czervionke et al 1978).

Collagen fibrils, microfibrils associated with elastin, basement membrane-like amorphous material and elastin represent morphologically identifiable connective tissue elements of the subendothelium. Intermediate and large vessels contain all four elements and these are exposed upon removal of the endothelium (Baumgartner and Muggli 1976).

Collagen, found in blood vessels, can be classified into four genetically distinct, structurally different subtypes (Barnes and MacIntyre 1979). Collagen Type III is found in the subendothelium of the young blood vessel and Type III and I are found in the media. Collagen Type IV and V are found in the basement membranes. Barnes and MacIntyre (1979) have demonstrated that platelets are much more reactive to Type III and Type I collagen than those types found in the basement membrane. This may serve as a controlling factor which regulates the size of the platelet plug to the degree of vessel injury. When the intima is

penetrated, collagen Type I is readily available to interact with the hemostatic elements of blood. Collagen can also activate blood factor XII (Niewiarowski et al 1965).

The adhesion of platelets to blood vessels has been reviewed by Baumgartner and Muggli (1976).

In addition to their role in vasoconstriction and dilatation, smooth muscle cells synthesize basement membrane, collagen, elastin and the proteoglycans of the extracellular space and these cells play a role in atherosclerosis (Ross and Harker 1976).

The Platelet: Ultrastructure

The human blood platelet is a disc-shaped, anucleate cell and is the smallest cellular element in circulating blood (less than 4 u in diameter) (White and Gerrard 1976). Platelets are produced in the bone marrow from mature megakaryocytes by a process involving the introversion of the surface membrane and the coalescence of the cytoplasmic membrane to form 2000 - 3000 platelets from each megakaryocyte (Crawford and Taylor 1971).

The platelet is divided into three regions: the peripheral zone, the sol-gel zone and the organelle zone (White 1971) in order to facilitate the understanding of relationships between structure and function. The peripheral zone consists not only of membranes and associated structures on the surface of the platelet but also those membranes lining the channels of the surface connected open canalicular system. It includes the exterior coat, the trilaminar unit membrane and the submembrane area containing specialized filaments which form the wall of the platelet and line channels of the surface connected

canalicular system. This is the site for transmission of stimuli triggering the platelet response for the adhesion and aggregation reactions (White and Gerrard 1978).

The sol-gel zone is the matrix of the platelet cytoplasm. This area contains actin microfilaments, structural microfilaments, a circumferential band of microtubules and glycogen. The fiber systems are in various states of polymerization and they support the discoid shape of unaltered platelets and provide a contractile system for shape change, pseudopod extrusion, internal transformation (centripetal movement of organelles following exposure to an aggregating agent) and secretion (White and Gerrard 1976).

The organelle zone consists of formed elements embedded in the sol-gel zone and include mitochondria, granules, dense bodies, elements of the dense tubular channel system and glycogen masses and particles. This area is involved in metabolic processes, the storage of enzymes, non-metabolic adenine nucleotides, serotonin (5HT) and calcium, and the biosynthesis of PGs (White and Gerrard 1978).

The membrane systems of the surface canalicular system and the dense tubular system serve as a platelet sarcoplasmic reticulum (White 1972).

Blood platelet actions include active transport of 5HT, adenosine and adenine, adhesion to biological macromolecules such as collagen and other components of the blood vessel wall, aggregation induced by ADP, adrenaline, calcium ionophores, acetylcholine, collagen, thrombin, vasopressin, immune complexes, PGs and 5HT, unmasking of membrane phospholipids such as factor III, active contraction, secretion from storage granules, PG synthesis and phagocytosis (Gordon and Milner

1976).

Platelets perform a number of functions along with their role in thrombosis and hemostasis. A description of the variety of activities in which the platelet is involved can be found in "Platelets: A Multidisciplinary Approach" (White and Gerrard 1978).

Prostaglandins and Thrombosis

The activity of PGs was discovered and reported by Goldblatt (1935) and von Euler (1936). They reported simultaneously that intravenous injections of human seminal plasma and extracts of the vesicular gland of sheep into small animals caused a fall in blood pressure and these extracts were found to stimulate a variety of smooth muscle preparations. These reports were preceded by that of Kurzok and Lieb (1930) who described the actions of human semen on isolated strips of non-pregnant human uterus.

It was not until 1957 that Bergstrom and Sjovall isolated two crystalline compounds from the vesicular gland of sheep and tested these for biological activity. These were labelled PGE and PGF and they possessed differences in biological activity. In 1964 Bergstrom et al, using homogenates of sheep vesicular gland, were able to generate PGE_2 from tritiated arachidonic acid (AA) (cis eicosa-5,8,11,14-tetraenoic acid) and the essential fatty acids were thereafter found to act as precursors of PGs.

Many important findings in the mid - 1960's caused a rapid increase in the knowledge of PGs. Piper and Vane (1969) reported that guinea pig lung released a substance that caused the isolated rabbit aorta to contract (rabbit aorta contracting substance, RCS). Vane (1971) reported

that indomethacin was a potent inhibitor of PG biosynthesis and that it blocked the formation of the substance that contracted aorta (Gryglewski and Vane 1972). Samuelsson (1965) demonstrated that all oxygens added to AA in PG biosynthesis were derived from molecular oxygen and further, that two oxygen atoms were derived from the same molecule. At this time a PG endoperoxide was envisioned.

Vargaftig and Zurnis (1973) reported that AA-induced platelet aggregation and PG synthesis and further, that in the course of aggregation, an activity was generated which caused rabbit aorta, rat stomach and rat colon to contract. Willis (1974) showed that when AA was incubated with a vesicular gland preparation, a potent aggregating substance was produced with an activity which disappeared in 6 - 10 minutes and could not be inhibited by aspirin.

Hamberg et al (1974c) reported on the structure of the two PG endoperoxide intermediates formed during the course of PG production. Both agents could induce platelet aggregation and were more potent than the PGs in contracting rabbit aorta strips. In an aqueous medium, activity was rapidly lost and the half life of the biological activity was estimated at 5 minutes. At this time the enzyme "fatty acid cyclo-oxygenase" was proposed for the enzyme catalyzing the oxygenation of the fatty acid at C-11 and C-15. There was however a problem in relating these endoperoxides to the biological activity of Piper and Vane (1969) as the half life of their biological activity was much shorter.

Hamberg et al (1974a) investigated the production of PGs from AA by aggregating platelets and concluded that there was activity with a half life of 41 $\pm$ 7 seconds - similar to the RCS of Piper and Vane.

Furthermore, the biosynthesis of this substance could be inhibited by indomethacin and it could be produced by using the PG endoperoxides as precursors. The compound was biosynthesized and trapped in methanol and, based on the knowledge of the precursor as demonstrated in biological assay studies, the chemical structure was determined and named TXA_2 (Hamberg and Samuelsson 1974, Hamberg et al 1974b).

The discovery of prostacyclin resulted from a search for different sites of PG, PG endoperoxide and AA metabolism in animals. In the study by Moncada et al (1976), microsomes were prepared from rabbit and pig aorta in which the adventitia had been removed. The microsomes were incubated with AA (1 - 10 ug/ml) and PGH_2 (0.1 - 1 ug/ml) and the products were studied using a bioassay cascade technique with strips of rabbit aorta, pulmonary artery, mesenteric artery, coeliac artery, vena cava, rat stomach strip, rat colon, chick rectum, guinea pig ileum and guinea pig tracheal chain. In this assay, control PG endoperoxides decomposed with a half life of 7 - 8 minutes at 22° C and this decomposition was accompanied by decreased activity on rabbit aorta. Also however, biological activity on the rat colon increased, indicating that other PGs, especially PGE_2 and $F_{2\alpha}$ were formed. When endoperoxides were incubated with the aorta microsomal preparation, the endoperoxide activity disappeared but was not accompanied by an increase in PGE_2 and $F_{2\alpha}$ and furthermore, no malondialdehyde was formed, suggesting another route of endoperoxide decomposition. The product of this incubation inhibited platelet aggregation with 30 times the potency of PGE_1 and 5 - 20 times the potency of PGD_2. Johnson et al (1976) reported the elegant work which led to the determination of the structure of prostacyclin. This work was centred around the compound 6-keto-$PGF_{1\alpha}$ which had been

reported by Pace-Asciak and Wolfe as early as 1971.

Cyclic Nucleotides and Thrombosis

A heat-stable factor mediating the action of adrenaline and glucagon on the activation of liver phosphorylase was reported in 1958 - adenosine-3',5'-cyclic monophosphate (cAMP) (Rall and Sutherland 1958, Sutherland and Rall 1958). At this time an enzyme capable of destroying the biological activity of cAMP was reported to occur in extracts of brain, heart and liver. The product of the enzymatic hydrolysis was 5'-adenosine monophosphate (5'-AMP) and the enzyme involved, cyclic nucleotide phosphodiesterase (PDE) (Rall and Sutherland 1958), was activated by magnesium ions and inhibited by caffeine. Cyclic AMP formation was mediated by the enzyme adenylate cyclase in the presence of ATP and Mg^{++} (Sutherland and Rall 1960). Determination of the site of action of cAMP required exhaustive study of the muscle phosphorylase action and led to the discovery of a protein kinase in an inactive form which could be converted to an active, phosphorylated form through cAMP (Krebs et al 1964).

The first evidence that cAMP affected platelets was from Marcus and Zucker (1965) who showed that exogenously added cAMP inhibited platelet aggregation. Wolfe and Shulman (1969) and Zieve and Greenough (1969) demonstrated that platelets contained the enzyme adenylate cyclase and Wolfe demonstrated that the platelet aggregation inhibitor PGE_1 stimulated this enzyme. Mills and Smith (1971) demonstrated synergism between subinhibitory doses of a PDE inhibitor (which also caused elevations in cAMP (Vigdahl et al 1971)) and PGE_1 - a stimulator of adenylate cyclase. This evidence suggested that elevations in cAMP

mediated the inhibition of platelet aggregation.

Ashman et al (1963) reported the occurrence of a second cyclic nucleotide, guanosine-3',5'-monophosphate (cGMP) and Jakobs et al (1974) reported the formation of cGMP in platelets. Goldberg et al (1973) proposed that cGMP modulated functions that had an opposite effect to those of cAMP, however, vasopressin caused effects on platelets which indicated that cGMP may also play an inhibitory role (Haslam 1975). A possible relationship of cGMP to the PG synthetic pathways has been suggested (Salzman 1978).

The physiological importance of cAMP as an intracellular mediator of platelet inhibition followed from reports that a physiological mediator of platelet inhibition, PGI_2, exerted its inhibitory effects on platelet function by stimulating the platelet-enzyme adenylate cyclase and, hence, through a cAMP mechanism (Gorman et al 1977, Tateson et al 1977). Earlier work by Mills and Macfarlane (1974) demonstrated that PGD_2, which is formed by platelets (Ali et al 1977, Oelz et al 1977) and inhibits platelet aggregation (Smith et al 1974b, Nishizawa et al 1975), stimulated platelet adenylate cyclase. PGI_2 was shown to be 10 times more active than PGD_2, 30 times more active than PGE_1 and 1000 times more active than its stable end product 6-keto $PGF_{1\alpha}$. In platelet aggregation studies, using PGH_2 as the aggregating agent, PGI_2 was 10 times more potent than PGD_2 and PGE_1 as an inhibitor of aggregation and, in this situation, 6-keto $PGF_{1\alpha}$ was much less active than these PGs (Johnson et al 1976).

Cyclic AMP appears to inhibit platelet aggregation at different levels (Salzman 1978) including inhibition of PG endoperoxide production (by inhibition of AA release or cyclo-oxygenase or both), inhibition of

the effects of endoperoxides or their products and inhibition of primary aggregation by ADP or other stimuli which is also reflected in cAMP inhibition of platelet adhesion to surfaces. A possible unifying mechanism for all these actions could be cAMP-induced alterations in cytoplasmic calcium levels. The effects of cAMP on the inhibition of platelets and the interaction with PGs on the activation of platelets has been discussed by Salzman (1978).

A further unifying factor for the roles of calcium and cyclic nucleotides as second messengers is the protein calmodulin. This protein was first reported by Cheung (1970) as an activator of the cyclic nucleotide PDE but has been found to modulate many cellular functions involving calcium (Klee et al 1980). This protein is found in platelets in large quantities and the importance of this protein to platelet function is demonstrated by the inhibitory effects of trifluoperazine (a relatively selective calmodulin inhibitor) on platelets (White and Raynor 1980).

Acetylsalicylic Acid (ASA) and Thrombosis

The use of salicylate for the relief of pain has been known since the time of Hippocrates when the bark of the willow was used for pain relief in childbirth. This effect of the bark of the common white willow (Salix alba vulgaris) was formally reported by Edward Stone to the Royal Society in 1763. The active ingredient, (salicin, a bitter glycoside), was reported 65 years later by Levoux. Various preparations of salicylic acid were reported thereafter by Pina (1838), Cahours (1844) and Kolbe and Lauteman (1860) and in 1875 Buss reported on the use of sodium salicylate as an antipyretic for rheumatic fever. In 1853 Charles

Frederick von Gerhardt reported on the successful preparation of the acetic acid ester of salicylic acid (ASA) but his work remained unnoticed for another 50 years. Felix Hoffman, a young staff chemist at Frederick Bayer and Company in Germany found the discarded compound in a literature search which stemmed from the desire to find a treatment for his father's rheumatoid arthritis. The agent was introduced after a successful clinical trial evaluation, Hoffman's father being one of the first to receive the drug (Taylor 1971, Flower et al 1980).

ASA, in small doses, was shown by Beaumont and Blatrix in the 1950's to increase the bleeding time in patients (Weiss 1971) and Quick demonstrated that sodium salicylate did not possess this effect. The inhibition effect of ASA on platelet adhesiveness to glass was reported in 1967 by Morris and the inhibitory effect on the second phase of ADP-induced aggregation was reported in 1968 by Zucker and Peterson. The most important current works, upon which the present hypothesis of the mechanism of action of ASA is based, were three papers published simultaneously in 1971 (Ferreira et al 1971, Smith and Willis 1971, Vane 1971). These works demonstrated the inhibitory effect of ASA on PG production by various systems and the hypothesis that inhibition of PG production accounted for the anti-inflammatory, antipyretic, analgesic and antiplatelet effects of ASA was put forward. It was difficult to support the role of PGs in platelet function at this time as the classical PGs did not cause platelet aggregation. This problem was overcome with the discovery of the proaggregatory PG endoperoxides and TXA_2 and the ability of ASA to inhibit the synthesis of these products (Hamberg and Samuelsson 1974).

The importance of the acetyl group on salicylic acid in causing the

hemostatic defect was first demonstrated by Quick (Weiss 1971). Weiss in 1968 reported that when volunteers ingested 1.5 g of ASA the blood salicylate levels reached maximum values at 6 hours after the dose and there was no detectable level at 24 hours (Weiss 1971). Despite this result, platelet ADP release was decreased after 24 hours and did not reach pretreatment levels until the 4th - 7th day after treatment (Weiss 1971). As the platelet lifespan was estimated at 7 - 10 days, this study suggested that ASA irreversibly damaged platelets and the hemostatic defect was not reversed until new platelets were produced. A biochemical explanation for this result was reported in 1975 (Majerus 1976). Roth and Majerus demonstrated that the labelled acetyl moiety of ASA covalently binds to a single platelet protein found in the particulate fraction that copurified with the cyclo-oxygenase enzyme. The time course of the acetylation followed that of the inactivation of platelets and, because platelets are anucleate and cannot synthesize new enzyme, the irreversible acetylation/inactivation of the enzyme lasts for the entire life span of the platelet. The time course of reappearance of nonacetylated enzyme followed the time course of new platelet production.

When viewed using the aggregometer, the effects of ASA on platelet function in response to low doses of collagen or ADP are quite dramatic. There are various possible reasons for the low success rate in clinical trials of ASA on thrombosis-related diseases. One of these reasons may be found in early reports (Zucker and Peterson 1970) that thrombin and high doses of collagen can induce full platelet aggregation and release in ASA-treated platelets. It has been suggested that ASA causes only a mild platelet defect which merely raises the threshold for platelet

aggregation (Majerus 1976). Secondly, the fact that the formation of the proaggregatory TXA_2 and the antiaggregatory PGI_2 is dependent on the cyclo-oxygenase enzyme suggests that ASA treatment may eliminate both influences and this may account for the apparent contradictory results both clinically and experimentally (Higgs et al 1980).

Current research is directed toward the hypothesis that ASA treatment may be manipulated to produce a net effect which is antithrombotic. The idea that platelet cyclo-oxygenase is more sensitive to the effect of ASA than that of endothelial cells is a matter of controversy (Burch et al 1978, Weksler 1980). Endothelial cells are able to regenerate the PGI_2 synthetic enzymes however, and can recover from the effects of ASA within 2 hours after removal of the drug according to Kelton et al (1978). In in vitro experiments, certain doses of ASA given to rabbits potentiated the antithrombotic effect of infused AA - presumably by blocking TXA_2 formation and in these same experiments the vessel wall cyclo-oxygenase quickly returned to normal whereas TXA_2 production remained impaired for longer periods (Amezcua et al 1978, Korbut and Moncada 1978). Higher doses of ASA, on the other hand, have been shown to potentiate thrombus formation in a rabbit venous thrombosis model (Kelton et al 1978).

In human studies for example, a single low dose of ASA (0.3 g) increases the bleeding time 2 hours after ingestion whereas a high dose (3.9 g) did not. TXA_2 formation was impaired for 168 hours on the high dose and bleeding time increased at 24 and 72 hours and returned to normal at 168 hours (O'Grady and Moncada 1978, Rajah et al 1978).

Other areas of potential pharmacological control of thrombosis include: the dietary manipulation of fatty acids to allow for the

optimal ratio of proaggregatory to antiaggregatory PGs (Needleman 1980), selective inhibition of TXA_2 synthesis, TXA_2 and PGH_2 antagonism, PGI_2 analogs that are stable and lack the usual side effects, cAMP PDE inhibition and calcium antagonism (Smith et al 1980).

Agents currently in use experimentally or clinically in the treatment of thrombosis include guanosine hydrochloride, propranolol and some steroidal anti-inflammatory drugs which inhibit the release of AA from membrane phospholipids; indomethacin, phenylbutazone and other nonsteroidal anti-inflammatory agents which inhibit the cyclo-oxygenase enzyme; various PGs which stimulate adenylate cyclase and dipyridamole which inhibits PDE - both of which lead to increased levels of cAMP; oral anticoagulants and heparin; propranolol and other beta blockers; clofibrate and halofenate which lower serum lipids and inhibit the second phase of platelet aggregation; ticlopidine which inhibits primary and secondary aggregation; vitamin E which may affect calcium flux and other agents whose mechanisms of action are not known (Packham and Mustard 1980).

Mills et al (1974) reported the effects of 2-propionyloxybenzoic acid (2-PBA) and 3-propionyloxybenzoic acid (3-PBA) on ADP and collagen-induced platelet aggregation. Cerskus (1978) examined 2-acetylbenzoic acid (ABA), 3-methylphthalide (3-MP) and 3-hydroperoxy 3-methylphthalide (3HMP) on platelet aggregation and compared these results with the effects of all these ASA-like agents on platelet PG synthesis. 2-PBA, which closely resembles ASA in structure, was a potent inhibitor of the second phase of platelet aggregation and PG synthesis, although it was less potent than ASA. ABA and 3-PBA did not inhibit PG synthesis or aggregation. 3HMP and 3-MP, although closely related to ASA

in structure were bicyclic compounds and had distinctive characteristics. 3HMP was the most potent inhibitor of total platelet aggregation and PG synthesis but appeared to inhibit platelet aggregation by additional mechanisms. 3-MP did not block platelet PG synthesis but was a potent inhibitor of both phases of aggregation. By demonstrating a wide variety of effects from a series of compounds with only slight structural differences from the ASA molecule, this work shed light on other possible mechanisms of action of salicylates and led to questions concerning the role of PG production in platelet aggregation.

2.2 Benzoic Acid Analogs and Thrombosis: Problems Examined

In the studies of ASA in the control of thrombosis, the following questions were addressed here:

1. In light of the fact that some of these analogs of ASA inhibit platelet aggregation without inhibiting platelet PG synthesis (3-MP) (Cerskus 1978) and that 3-PBA appears to augment platelet aggregation and the production of proaggregatory substances by platelet microsomes (Mills et al 1974, Cerskus 1978) is it possible to separate the effects of these agents on collagen-induced aggregation and ADP-induced second phase aggregation from the effects on the simultaneous release reaction? This would provide further information concerning the central role of PG production in platelet aggregation and its relation to the release reaction.

2. As inhibition of total PG synthesis does not explain the inhibition of platelet aggregation by all agents examined, and certain PG synthesis inhibitors do not display typical NSAID effects in platelet aggregation studies (3HMP), do these agents inhibit platelet aggregation through other likely mechanisms such as inhibition of TXA_2 synthesis or elevation of cAMP through the inhibition of platelet PDE?

3. Is it possible to separate antiplatelet PG and/or antiplatelet aggregation activity from effects on PGI_2 activity produced by aorta rings using these analogs? If the balance of proaggregatory PG

endoperoxides and antiaggregatory PGI_2 is an important regulator of thrombosis, agents with these characteristics would be likely candidates as antithrombotics.

4. Do these agents inhibit thrombus formation in an in vivo rat model of thrombosis? If so, can the effects be explained by any of the known mechanisms of action of ASA or any of the above information or, do other mechanisms of action appear to be operating?

2.3 Effects of Benzoic Acid Analogs on Platelets In Vitro

2.3.1 Effects of Benzoic Acid Analogs on Platelet Aggregation and the Release Reaction

2.3.1.1 Introduction

The process of platelet aggregation is studied using platelet-rich plasma (PRP) and a platelet aggregometer. PRP is prepared by gentle centrifugation of anticoagulated whole blood to remove red and white cells, leaving the platelets suspended in the plasma. A portion of PRP, in a tube, is placed in the path of a beam of light and is stirred so that the cloud of platelets blocks much of the beam of light from reaching a photocell on the other side of the transparent tube. When an aggregating agent is added, the platelets clump together allowing more light to pass through. A tracing representing the amount of light reaching the photocell provides a continuous record of the course of platelet aggregation (Zucker 1980). Through the use of an infrared light source for measuring aggregation, the firefly luciferin-luciferase enzyme system suspended in the PRP to measure adenosine triphosphate (ATP) and photocells positioned at right angles to each other, it is now possible to measure the platelet secretion of ATP through luminescence, simultaneously, with the course of platelet aggregation (Feinman et al 1977).

The ability to measure platelet aggregation and the discovery of the stimulating effect of ADP (Born 1962) on platelets provided the

foundation for subsequent understanding of platelet physiology and biochemistry and the effects of drugs on platelets. Reports that the effect of ADP could be inhibited by such compounds as ATP, adenosine monophosphate (AMP) (Born 1962) and adenosine (Clayton et al 1963) suggested the presence of a specific platelet receptor for ADP and this is an area of current study (Mills and Lipson 1981). Various candidates for the ADP receptor include platelet membrane glycoproteins, ecto ATPase and adenylate cyclase (Philp 1981). A nucleoside diphosphate kinase has also been proposed as the ADP receptor (Mustard and Perry 1972) but this idea has been challenged (Lips and Sixma 1977, Mills and Lipson 1981).

Platelets require various cofactors in order to aggregate to ADP including divalent cations (Holmsen et al 1977, Lages and Weiss 1980) and fibrinogen (Cross 1964, McLean et al 1964, Zucker 1980). The importance of fibrinogen was confirmed by Mustard et al (1975) and the fact that ADP leads to primary aggregation only in the presence of fibrinogen suggests that it serves as a glue, binding platelets to one another. A congenital lack of fibrinogen results in a prolonged bleeding time in humans (Zucker 1980).

An important controversy in platelet aggregation studies involves the choice of citrate or heparin as the anticoagulant. ADP does not induce the second wave of aggregation in heparin anticoagulated PRP although this is known to occur when citrate is used (Mustard et al 1975). Although heparin may cause certain artificial characteristics in platelet aggregation studies, Lages and Weiss (1980) have shown that platelet secretion in response to ADP is maximal over a narrow range of Ca^{++} and Mg^{++} concentrations - a range provided by citrate

anticoagulation. Collagen-induced secretion occurs by Ca^{++} dependent and independent ways (Heptinstall and Taylor 1979).

Other conditions affecting ADP-platelet aggregation in general have been reviewed by Packham et al (1978), Breddin et al (1980) and Philp (1981).

Collagen-induced aggregation of blood platelets (Zucker and Borelli 1962) is dependent upon the initial adherence of platelets to collagen fibers and this adherence is not dependent on extracellular calcium (Spaet and Zucker 1964). In order to induce platelet aggregation collagen must meet certain structural requirements (Philp 1981) including the native triple helix, presence of free amino groups and polymerization in the fibrillar form. The importance of the quaternary structure is a matter of debate (Muggli 1978).

Baker et al (1959) isolated 5HT and ATP from platelet cytoplasmic granules and Haslam (1964) showed that thrombin induced the release of ADP from platelets. Holmsen (1965) using a ^{32}P label demonstrated two pools of adenine nucleotides in platelets, a metabolic pool and a releasable pool. Platelet secretion is the energy dependent specific release of dense and alpha granule bound substances with the retention of mitochondria, membranes and cytoplasmic substances. Secretion should be distinguished from the release of AA oxidation products, hypoxanthine, lactate and the drug induced 5HT depletion (Holmsen and Weiss 1979). The dense granules of platelets are so termed because they show the greatest density in sucrose density gradient centrifugation and also appear as electron dense spots on electron micrographs. They contain 5HT, calcium, ATP, ADP and pyrophosphate. Human platelets contain 4.6-7.0 umoles ATP/10^{11} cells and 2.4-4.0 umoles ADP/10^{11} cells

(Holmsen et al 1977). The release of the dense granule constituents requires a different concentration (usually lower) of platelet activating agents than the alpha granules (Day and Holmsen 1971). The alpha granules may be heterogeneous and contain platelet specific proteins such as platelet factor IV and beta thromboglobulin, growth factors, permeability factors, chemotactic factors, bacteriocidal factors, fibrinogen, factor V and glycoprotein. Acid hydrolases are not found in the alpha or dense granules but are found in vesicles which may be derived from the Golgi apparatus of the megakaryocyte and are also releasable. Most substances found in platelet alpha granules and vesicles are synthesized and packaged in the megakaryocyte (Holmsen and Weiss 1979).

The secretion of fibrinogen from platelets plays an important role in thrombus formation. Fibrinogen released from the alpha granules of platelets is gradually converted to fibrin as thrombin becomes available in the process of thrombus formation and this fibrin in turn stabilizes the platelet plug (Sixma et al 1978). The events involved in the release reaction have been summarized by MacIntyre (1976).

The mechanism of action of various platelet aggregating agents were classified by Packham et al (1977). They concluded that different agents induce platelet aggregation by:

(i) causing the release of ADP

(ii) causing the formation of PG endoperoxides and TXA_2 from AA. These agents can cause aggregation independently of released ADP although, with intact platelets, they also induce ADP release. Collagen-induced aggregation appeared to involve these two mechanisms.

(iii) primary aggregation mechanisms that occur without or before

secondary aggregation, release and the formation of PG endoperoxides. ADP, 5HT, adrenaline and vasopressin cause primary aggregation.

(iv) other mechanisms which appear to occur concurrently with ADP release and PG endoperoxide formation. Thrombin at high doses can cause extensive platelet aggregation and release when both these mechanisms are blocked. Chignard et al (1980) suggested that platelet activating factor (PAF) was a likely mediator of this mechanism. In this report AA was classified as an agent which, like collagen, caused aggregation together with release, but also caused aggregation in thrombin-treated platelets and hence independent of release.

The proposed mechanism for platelet aggregation and release in the presence of high concentrations of collagen has been modified based on a report by Kinlough-Rathbone et al (1980). In this study, high dose collagen aggregation was not inhibited by combinations of creatine phosphate/creatine phosphokinase (CP/CPK a system which removes ADP) and a NSAID. Collagen appeared to activate platelets through a mechanism independent of PGs and ADP but dependent on calcium.

The relation of the release reaction to platelet aggregation has been further studied using the lumi-aggregometer of Feinman et al (1977) which measures the simultaneous release of ATP and platelet aggregation (Charo et al 1977a, 1977b). Charo et al (1977a) classified platelet release of ATP according to one of two mechanisms:

(i) release lags behind aggregation, is dependent on aggregation and is inhibited by indomethacin. ADP and adrenaline activate platelets only by this aggregation mediated mechanism.

(ii) release begins at the same time as aggregation, occurs in the absence of aggregation and is not blocked by indomethacin. Thrombin and

ionophore A23187 activate platelets by either the aggregation mediated mechanism or this direct activation mechanism. Collagen appears to activate platelets only by the direct method and most aggregation is due to released substances from collagen-activated platelets. The endoperoxides and TXA_2 are able to induce aggregation without secretion of nucleotides and higher concentrations can induce secretion independent aggregation (Charo et al 1977b). These results are generally in line with and an extension of the classification by Packham et al (1977).

Kinlough-Rathbone et al (1976) demonstrated that AA caused aggregation in platelets depleted of their secretory granules. Based on these results, the commonly held view that second phase aggregation and other forms of aggregation (such as AA-aggregation) were dependent on platelet secretion of aggregating substances was questioned. The idea that platelet aggregation and secretion are parallel events provides an explanation for these results. Further evidence for the separation of release from second phase aggregation was provided by Rao et al (1980) who were able to reverse the inhibitory effect of ASA on biphasic ADP and thrombin-induced aggregation and on AA-induced aggregation, but not release, by preincubation with adrenaline before the addition of the aggregating agent.

Weiss and Lages (1981) however provided further evidence of the central role of released ADP in platelet aggregation in a study of 12 patients with various types of platelet storage pool deficiency (SPD). AA and PGG_2-induced platelet aggregation were abnormal in many SPD patients and there was a close correlation between the aggregation response to AA and the amount of endogenous ADP. These authors conceded

that these results did not resolve the evidence of conflicting studies cited, including those reported here (Kinlough-Rathbone et al 1976, Charo et al 1977b), but suggested that the contribution of released ADP to aggregation may occur only within the microenvironment of the platelet to activate it. In this way small amounts of granule-derived ADP could mediate aggregation in the absence of measurable secreted ADP.

The role of released ADP is, at present, controversial in secondary aggregation (Lages and Weiss 1980, Huang and Detwiler 1981), collagen aggregation (Weiss 1975, Nuhn 1979) and AA, PG endoperoxides, and thromboxane mediated aggregation (Weiss and Lages 1981).

The synergistic effect of platelet aggregating agents is another area of current research in understanding platelet function (Packham et al 1977, Grant and Scrutton 1980, Huang and Detwiler 1981) and may prove to be an important area for understanding thrombus formation and the effects or lack of effects of antiplatelet agents.

The pharmacological inhibition of platelet aggregation has been reviewed recently (Smith et al 1980, Packham and Mustard 1981). Agents can inhibit platelet aggregation by a variety of mechanisms (Packham and Mustard 1981) and, to a certain extent, these mechanisms are reflected in the pattern of inhibition viewed in in vitro platelet aggregation studies using the platelet aggregometer. Although biphasic aggregation may be an artifact, it is believed to reflect important physiological functions and is especially important as a parameter of in vitro platelet function dependent on PG synthesis (Charo et al 1977b).

Agents which inhibit PG synthesis through inhibition of the cyclo-oxygenase enzyme inhibit only the second phase of ADP-induced

aggregation in citrated PRP (McIntyre and Philp 1977). This effect has also been reported for a thromboxane receptor antagonist SQ26536 (Harris et al 1981). Although the importance of ~~TXA_2~~ in second phase aggregation and release is under study (Gorman et al 1977, Needleman et al 1977) Kakar and Westwick observed that aggregation occurred with AA, collagen or adrenaline in the presence of thromboxane synthesis inhibition (Smith et al 1980). Platelet secretion of 5HT and ATP were reduced in this situation. Suda and Aoki (1981) have demonstrated that the calmodulin inhibitor N-(6-amino)-5-chloro-1-napthalene sulfonamide (ω-7) inhibited second phase aggregation and release by ADP and aggregation by collagen, adrenaline and other aggregatory agents. Vitamin E inhibits second phase platelet aggregation (Kurokawa et al 1971), possibly through interference with the movement of internal calcium (Butler et al 1979). White (1971) demonstrated that cytochalasin B partially inhibits second phase aggregation and release by inhibiting the platelet contractile system. Propranolol and other beta blockers which may inhibit phospholipase A_2 (Weksler et al 1977, Vanderhoek and Feinstein 1979) and clofibrate and halofenate, of which the mechanisms of inhibition are less well defined (Robinson and LeBeau 1967), also inhibit the second phase of platelet aggregation specifically.

Apart from the interference with specific cofactors of ADP-induced aggregation such as calcium and fibrinogen, membrane stabilization and specific receptor antagonists, a number of agents inhibit the release and aggregation of platelets to collagen, ADP and adrenaline completely. Agents that cause increases in intracellular cAMP have been shown to inhibit platelet aggregation completely. Adenylate cyclase is stimulated by various PGs including PGI_2, PGE_1 and PGD_2 (Gorman et al 1977, Mustard

and Packham 1978) all of which are potent inhibitors of platelet aggregation (Kloeze 1970, Smith et al 1974b, Moncada et al 1976). Inhibitors of cAMP PDE activity also inhibit platelet function and these include caffeine (Mills and Smith 1971) and dipyridamole and analogs (Philp et al 1973, McElroy and Philp 1975). Calcium antagonists such as nifedipine completely inhibit platelet aggregation as do certain steroids such as methylprednisone (Pierce et al 1974) which may inhibit phospholipase A_2 as part of their mechanism of action (Flower 1978). Combinations of deoxy-D-glucose, which inhibits glycolysis, with either antimycin or oligomycin, which inhibit oxidative phosphorylation, completely inhibit platelet aggregation (Murer et al 1967). Ticlopidine inhibits primary platelet aggregation (O'Brien et al 1978) and although the mode of action is unclear, this drug enhances the effects of PGI_2 (Johnson and Heywood 1979) suggesting some interaction with platelet cyclic nucleotides (see Mustard and Packham 1978, Packham and Mustard 1981 and Philp 1981).

Mills et al (1974) reported the study of a series of ASA analogs on collagen and ADP-induced platelet aggregation. 2-PBA displayed the same inhibitory characteristics as seen with ASA in the aggregometer, but at a reduced potency. By moving the 2-propionyloxy group to the 3-position all inhibitory activity was abolished and the second phase of aggregation was actually increased. This study demonstrated the importance of the position of the acetoxy group on the ASA molecule and also demonstrated that slight variations in this group were permissible for the inhibitory activity to be retained.

Cerskus (1978) demonstrated that a change in the 2-position substituent from an acetoxy to acetyl (2-acetylbenzoic) caused a marked

reduction in potency of the agent but led to a type of inhibition of platelet function which was not typical of ASA-like drugs. Because this agent existed in ring and chain tautomeric forms, 3-MP was synthesized due to its structural similarity to the ring form and it was tested on platelet aggregation in an attempt to characterize the activities of the ring form of ABA. This agent inhibited both phases of ADP-induced platelet aggregation and was more potent than ABA in this regard.

A hydroperoxy analog of 3-MP, 3-hydroperoxy-3-methylphthalide (3HMP) was reported to inhibit platelet aggregation with an even greater potency than 3-MP and like 3-MP inhibited both the first and second phases of platelet aggregation (Cerskus 1978, Cerskus and Philp 1981).

Based on the reports by Charo et al (1977a, 1977b) that platelet aggregation and release were separate, parallel events and, in view of the widely divergent effects of this group of ASA-like agents, we reassessed the effects of these agents on platelet aggregation in relation to the release reaction as reflected in the simultaneous release of ATP by aggregating platelets.

2.3.1.2 **Methods**

Blood Collection

Whole blood was obtained from healthy male and female donors, age 20-50 years, who had not taken any medication (excepting oral contraceptives) for at least 1 week. Donors were paid a small fee for their inconvenience. Blood was collected by venipuncture in the area of the antecubital fossa using a plastic disposable syringe and a 20 gauge, 1 inch needle. Blood was anticoagulated with 3.8% trisodium citrate (McArthur Chemical Co., Ltd., Montreal) by mixing, in a ratio of 1:9 parts blood, in siliconized (Siliclad, Clay Adams, Canlab Laboratory Supplies Ltd., Toronto) test tubes or 50 ml polycarbonate centrifuge tubes.

Preparation of platelet-rich plasma (PRP)

Whole blood, anticoagulated as described above, was centrifuged at 967 x g for 3 minutes in a clinical centrifuge. The PRP supernatant was removed using a 5 ml siliconized glass syringe to which an 18 gauge needle and 5 cm PE 160 tubing was attached. The PRP was collected into siliconized 15 ml conical centrifuge tubes. Platelet counts were done for each donor by the method of Wright and Scholar (1941) using a Spencer Bright Line hemocytometer (American Optical Instruments Co., N.Y.) and a platelet dye described in Appendix III. Counts were in the range of $300-500 \times 10^3/mm^3$.

Study of drug effects on ADP and collagen-induced aggregation and simultaneous release of platelet ATP

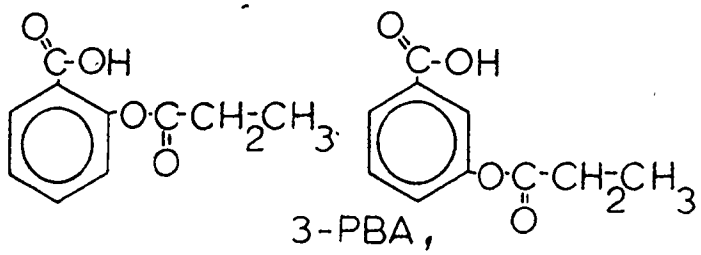
PRP was divided into 460 ul portions and placed into 0.312 inch x 2 inch cuvettes (Chronolog Corp., Haverton, PA.). PRP was then allowed to stand at room temperature for at least 30 minutes. ADP and collagen-induced platelet aggregation were studied by the turbidometric method of Born (1962) and this and the simultaneous release of platelet ATP were measured using a Chronolog Model 400 Lumi aggregometer (Chronolog Corp., Haverton PA.) and recorder (Rikadenki Model B141). PRP with 20 ul of Tris-HCl buffer (0.05 M pH 7.4) or drug made up in Tris-HCl buffer was incubated at 37° C for 5 minutes. Firefly extract suspension, 50 ul, consisting of 50 mg of the soluble extract of dried firefly lanterns (FLE 50, Sigma Chemical Co., St.Louis) in 3 ml of double distilled water, was added after 3.5 minutes. The suspension contained the necessary cofactors for the ATP assay and gave a final concentration of 7.4 mM potassium arsenate and 3.0 mM $MgSO_4$. A small piece of magnetized 18 gauge needle stylet wire was used as a stir bar. It was added at 4.5 minutes and a baseline was established for 0.5 minutes afterwhich the aggregating agent was added. All studies took place at 37° C with stirring (1000 rpm). Calibration of the aggregometer was done automatically with reference to a sample of PRP and platelet poor plasma (PPP) placed in the aggregometer and required only a simple adjustment of the recorder. ATP release calibration was performed by the addition of a standard amount of ATP and using one setting of the sensitivity for release which usually resulted in release curves about 75% of the height of aggregation curves (Charo et al 1977c). In this

system an infrared light (IR) source is simultaneously passed through both the PRP and the PPP samples and the light transmission is detected by IR sensitive photodiodes. The difference is determined through digital techniques and the resultant voltage is recorded as the curve of platelet aggregation. In the presence of ATP, the added firefly extract becomes luminescent and a sensitive photomultiplier tube positioned at right angles to the aggregation channel light path detects luminescence and provides a voltage output which is directly related to the amount of ATP released. This voltage is recorded as the ATP secretion curve. ADP (Sigma Chemical Co., St. Louis) was added in volumes up to 20 ul using an Alga Micrometer Syringe (Burroughs Wellcome and Co., England). Drugs and collagen (General Biochemicals, Chagrin Falls, Ohio, Appendix III) were added using 10 and 20 ul Oxford pipettes (Oxford Laboratories, California). Aggregation was followed for at least 5 minutes afterwhich 20 ul of stock ATP solutions (final concentration 1.5 uM) was added to calibrate the ATP curves. The first phase height of ADP aggregation and the five minute maximum height of both ADP and collagen curves and the ATP release curves were compared to control curves every 2 runs.

Study of drug effects on arachidonic acid aggregation

Arachidonic acid (AA) aggregation was measured in a Born aggregometer (Royal College of Surgeons, London, England) equipped with a Rikadenki Model B141 recorder. The aggregometer was standardized to read 10% light transmission for PRP and 90% light transmisson for PPP. PRP was divided into 350 ul portions and treated as above without the addition of firefly extract. AA (Grade I, Sigma Chemical Co., St. Louis) was dissolved in 20 ul ethanol and then saline to a final concentration

Fig. 1 Chemical structures of the benzoic acid analogs studied here.

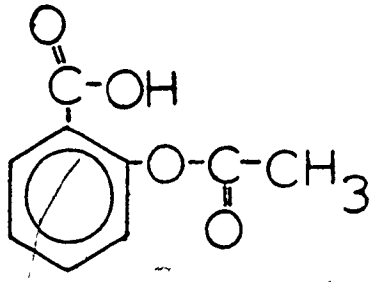


2-PBA

2-propionyloxybenzoic acid

3-PBA,

3-propionyloxybenzoic acid

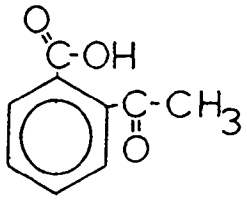


ASA

2-acetylsalicylic acid

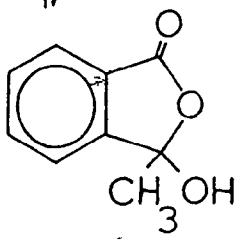
or

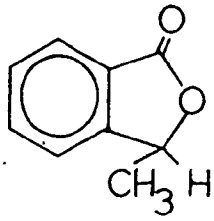
2-acetoxybenzoic acid



ABA

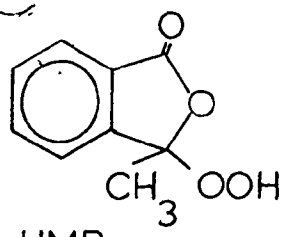
2-acetylbenzoic acid





3-MP

3-methylphthalide



3-HMP

3-hydroperoxy-3-methylphthalide

of 38 uM. The final ethanol concentration did not exceed 0.05%. Drugs were added in a volume of 20 ul for a final concentration of 0.5 mM. AA was added in a volume of 10 ul for a final concentration of 1 mM. The 5 minute maximum height of platelet aggregation in the presence of drugs was compared with Tris controls.

Chemicals

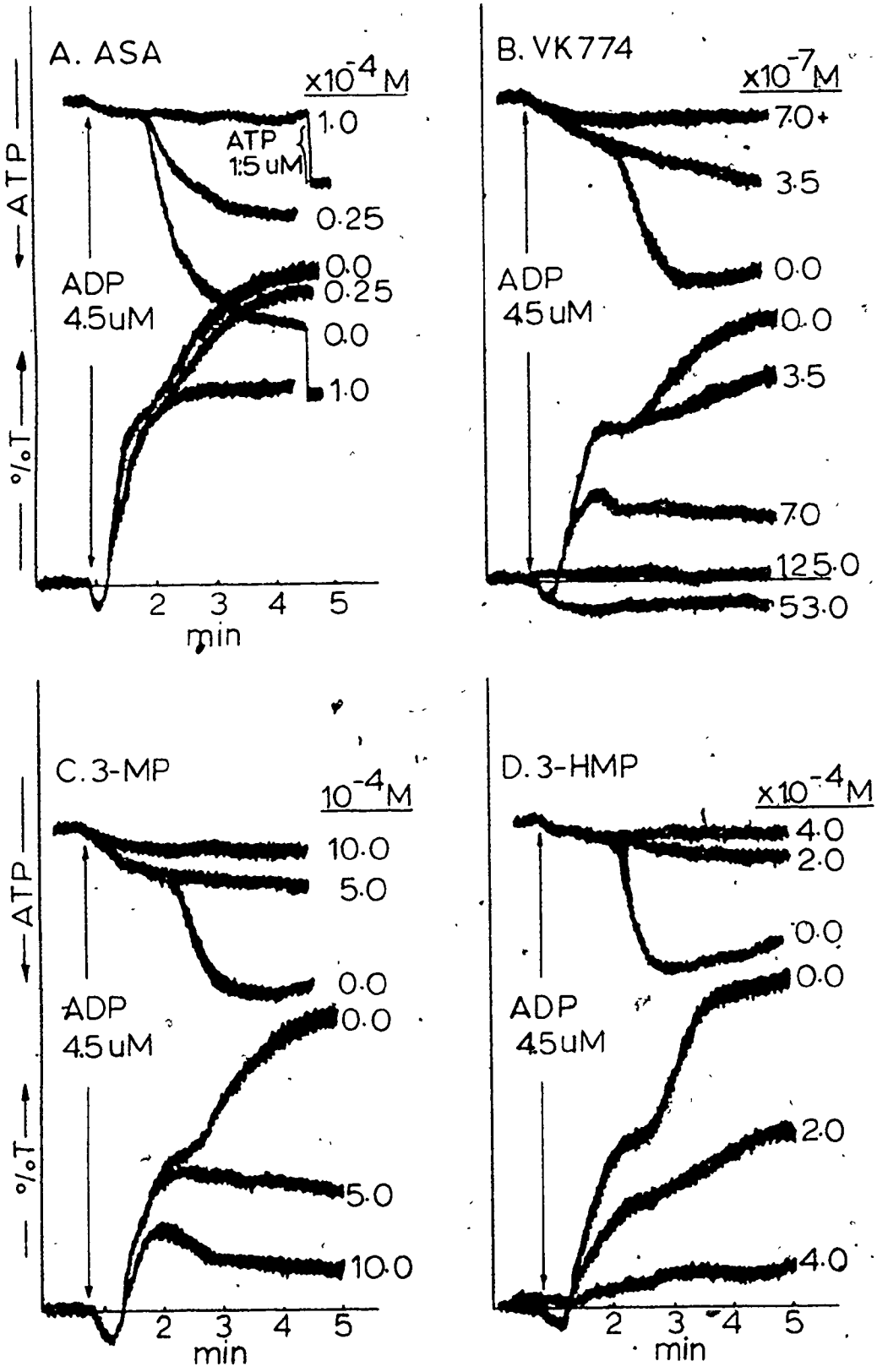
The chemical structure of the compounds used are shown in Fig. 1. ASA was obtained from the Sigma Chemical Co., St. Louis. ABA was obtained from the Aldrich Chemical Co., Montreal. 3-MP, 2-PBA and 3-PBA were synthesized in this laboratory under the supervision of Dr. M. Hirst (Dept. of Pharmacology and Toxicology, the University of Western Ontario, London, Ontario). Unless otherwise noted drugs were dissolved in Tris-HCl buffer (0.05 M, pH 7.4). 3-MP was initially dissolved in dimethylsulfoxide (DMSO, Anachemia Chemicals Ont.) and then Tris-HCl. DMSO final concentration did not exceed 0.2%. Controls were performed with vehicle alone and drug solutions were always prepared immediately prior to use (see Appendix II).

2.3.1.3 **Results**

The firefly luciferin-luciferase system used in the ATP-release experiments proved satisfactory for measurements of the amount of ATP released under these conditions and caused negligible inhibition of platelet responsiveness to ADP or collagen. This enzyme system gave a linear response to ATP standard stock solutions over a concentration range of 0.5-6 uM and was not inhibited by the agents studied at the concentrations used in these experiments. Typical aggregation and

Fig. 2 Typical effects of ASA, VK774, 3-MP and 3HMP on ADP-induced platelet aggregation and release of ATP.

For each set of tracings, the top tracings represent the release of ATP and the bottom tracings represent the simultaneous platelet aggregation.
Drugs were preincubated with platelets for 5 min before the addition of ADP. VK774 represents the effects of a typical PDE inhibitor.


A. ASA
x10⁻⁴M
1.0
ATP
1.5 uM
0.25
0.0
ADP
4.5uM
0.25
0.0
1.0
ATP
%T
2 3 4 5
min
B. VK774
x10⁻⁷M
7.0+
3.5
0.0
ADP
45 uM
0.0
3.5
7.0
125.0
53.0
C. 3-MP
10⁻⁴M
10.0
5.0
0.0
ADP
4.5uM
0.0
5.0
10.0
ATP
%T
2 3 4 5
min
D. 3-HMP
x10⁻⁴M
4.0
2.0
0.0
0.0
ADP
45uM
2.0
4.0
2 3 4 5
min

release curves and the effects of ASA are shown in Fig. 2. ASA, over the concentration range tested, inhibited the second phase of ADP-induced aggregation and completely inhibited ATP release, but did not inhibit first phase or primary aggregation (Fig. 2A). For comparison, the effects of VK774, a potent PDE inhibitor (McElroy and Philp 1975) are shown in Fig. 2B. In contrast to ASA, VK774 inhibited second phase aggregation, first phase aggregation and shape change of ADP-stimulated platelets. Release of ATP was not detected when second phase aggregation was inhibited.

ASA was the most potent inhibitor of ADP second phase aggregation and the effects on the release of ATP parallel the effects on second phase aggregation (Fig. 3). This is demonstrated as only partial inhibition of the total aggregation curve. 2-PBA had less potent but similar qualitative effects to ASA. Moving the propionyloxy group to the 3-position, (3-PBA), not only resulted in a loss of platelet inhibition activity but also in a slight potentiation of platelet ATP release. Substitution of a 2-acetyl group for the 2-acetoxy group of ASA (ABA), abolished all platelet inhibitory effects at the concentration tested.

3-MP was designed to resemble the ring tautomeric form of ABA. It inhibited both first phase and second phase aggregation and the release of ATP in a concentration dependent manner (Fig. 2C,4) over this concentration range and had effects qualitatively similar to VK774. 3HMP, the photo-oxidation product of 3-MP was a more potent inhibitor of first phase and second phase aggregation and ATP release than 3-MP and it exhibited a steep dose-response curve for the inhibition of aggregation and release as previously reported (Cerskus 1978) (Fig. 2D,4). The effect of ASA on ATP release and second phase aggregation

Fig. 3 Dose-response effects of ASA, ABA, 2-PBA and 3-PBA on ADP-induced platelet aggregation and release of ATP.

A. First phase height

B. Total 5 min height of aggregation curve

C. ATP release

Drugs were preincubated with platelets for 5 min before the addition of ADP.
% Inhibition of release of ATP by 3-PBA at 10^{-4} M is -28 $\pm$ 12 (release is actually augmented).

For each point n=4.

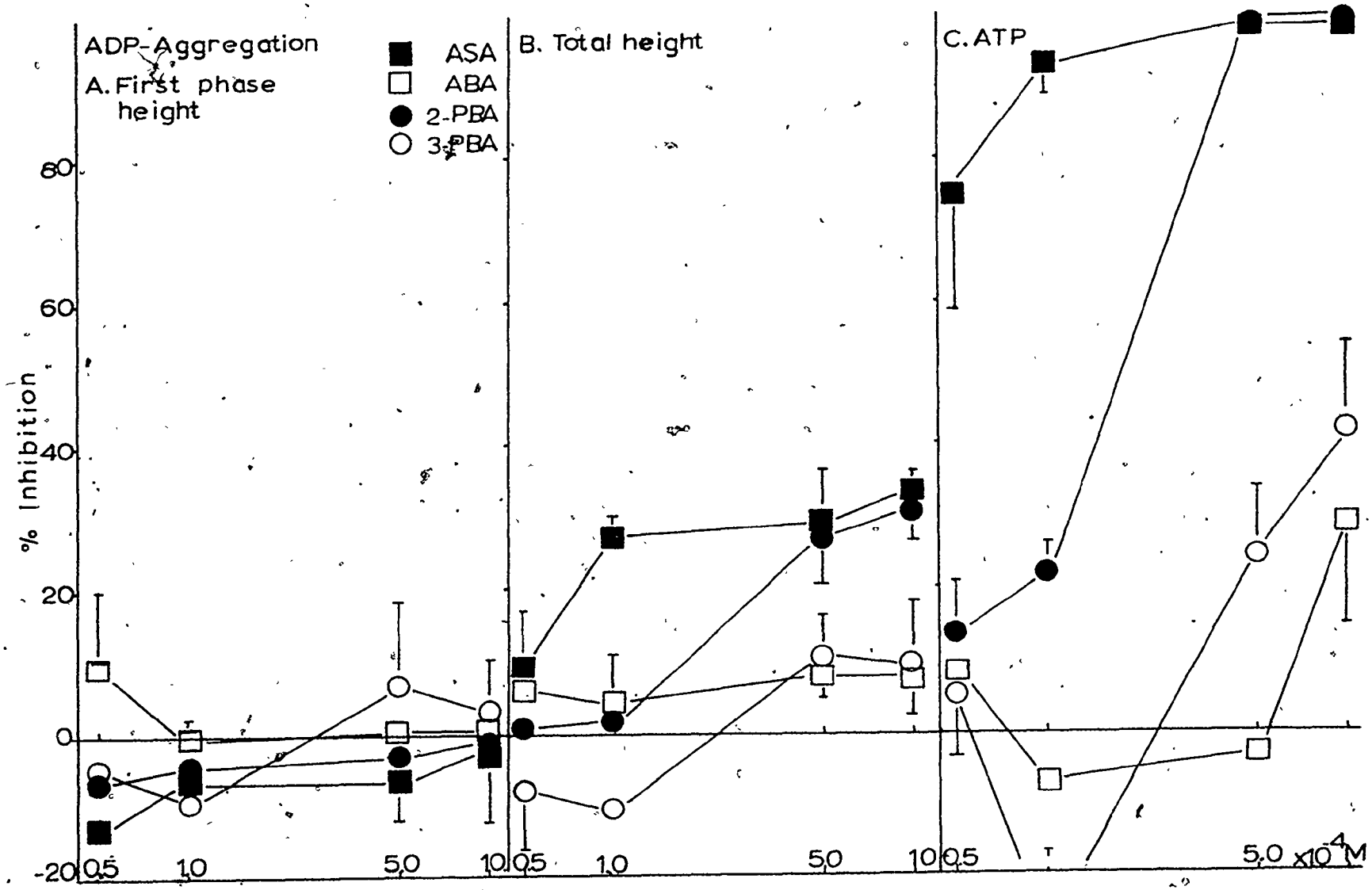

ADP-Aggregation
A. First phase height
B. Total height
C. ATP
ASA
ABA
2-PBA
3-PBA
% Inhibition
60
40
20
0
-20
0.5
1.0
5.0
10
5.0 x10⁻⁴M

Fig. 4 Dose-response effects of ASA, ABA, 3-MP and 3HMP on ADP-induced platelet aggregation and release of ATP.

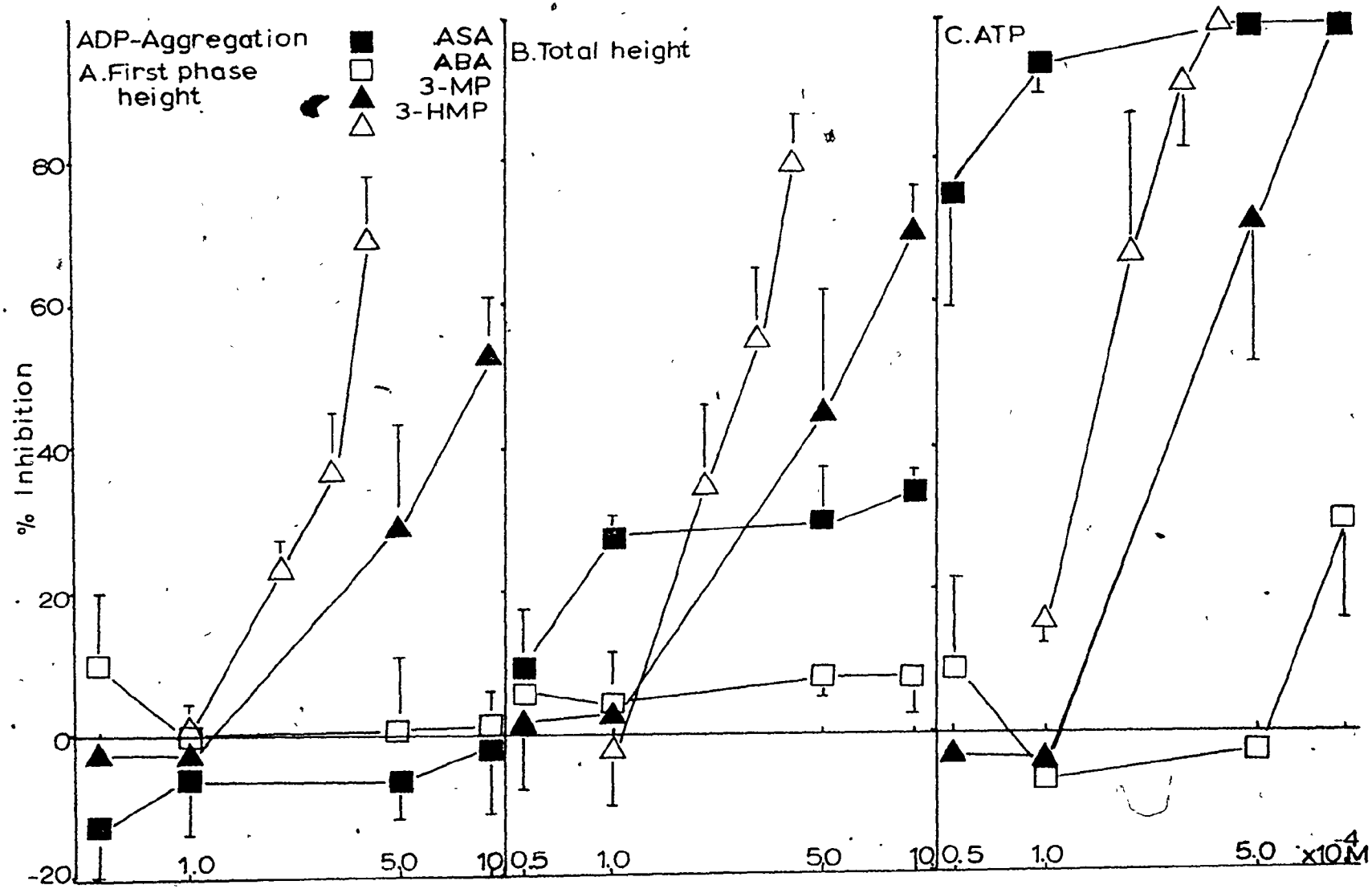
A. First phase height

B. Total 5 min height of aggregation curve

C. ATP release

Drugs were preincubated with platelets for 5 min before the addition of ADP.

For each point n=4.


ADP-Aggregation
A.First phase height
ASA
ABA
3-MP
3-HMP
B.Total height
C.ATP
% Inhibition
60
40
20
0
-20
1.0
5.0
10
0.5
1.0
5.0
10
0.5
1.0
5.0
x10⁻⁴M

occurred at a lower dose than did 3HMP inhibition of aggregation.

In contrast to ADP-induced aggregation, the release of ATP coincided with the onset and course of collagen-induced aggregation (Fig. 5A,B). The effects of ASA on collagen-induced aggregation were similar to the effects on second phase ADP-induced aggregation and it was again the most potent inhibitor (Fig. 6). 2-PBA, 3-MP and 3HMP (Fig. 6) were less potent than ASA and ABA and 3-PBA had no significant effects on collagen-induced platelet aggregation at these concentrations. There was no separation of collagen aggregation inhibition and collagen-ATP release in this study.

A brief study was carried out to assess the effects of these agents on AA-induced platelet aggregation. All agents were studied at $5x10^{-4}$ M final concentration except 3HMP which was studied at $3x10^{-4}$ M and aggregation was induced with 1 mM AA. ASA was the most potent inhibitor of AA aggregation (90.0 $\pm$ 8.0% inhibition) and 2-PBA and 3HMP demonstrated similar inhibitory activity (61.0 $\pm$ 3.2% and 59.0 $\pm$ 22.0% inhibition respectively). 3-MP was a weaker inhibitor (29.0 $\pm$ 13.7% inhibition) and ABA (4.0 $\pm$ 10.0% inhibition) and 3-PBA (3.5 $\pm$ 2.2% inhibition) had no significant effect here. Due to interactions between the firefly enzyme system and arachidonic acid, ATP release was not studied here.

2.3.1.4 **Discussion**

The effects of this group of ASA-like drugs on collagen and ADP-induced platelet aggregation were examined to confirm the profile of effects of Mills et al (1974) and Cerskus and Philp (1981) and to extend their findings by also examining the simultaneous release reaction. All

Fig. 5 Typical effects of benzoic acid analogs on collagen-induced aggregation and ATP release and AA-induced aggregation.

A. 2-PBA and B. 3-MP on collagen-induced aggregation and ATP release.
For each set of tracings, the top tracings represent the release of ATP and the bottom tracings represent the simultaneous platelet aggregation.
Drugs were preincubated with platelets for 5 min before the addition of collagen.

C. ASA on AA-induced platelet aggregation

D. Platelets aggregating to AA generate a substance(s) which causes ASA-treated platelets to aggregate.
100 ul of AA-induced aggregating platelets was transferred to 350 ul of ASA-treated platelets and aggregation took place.

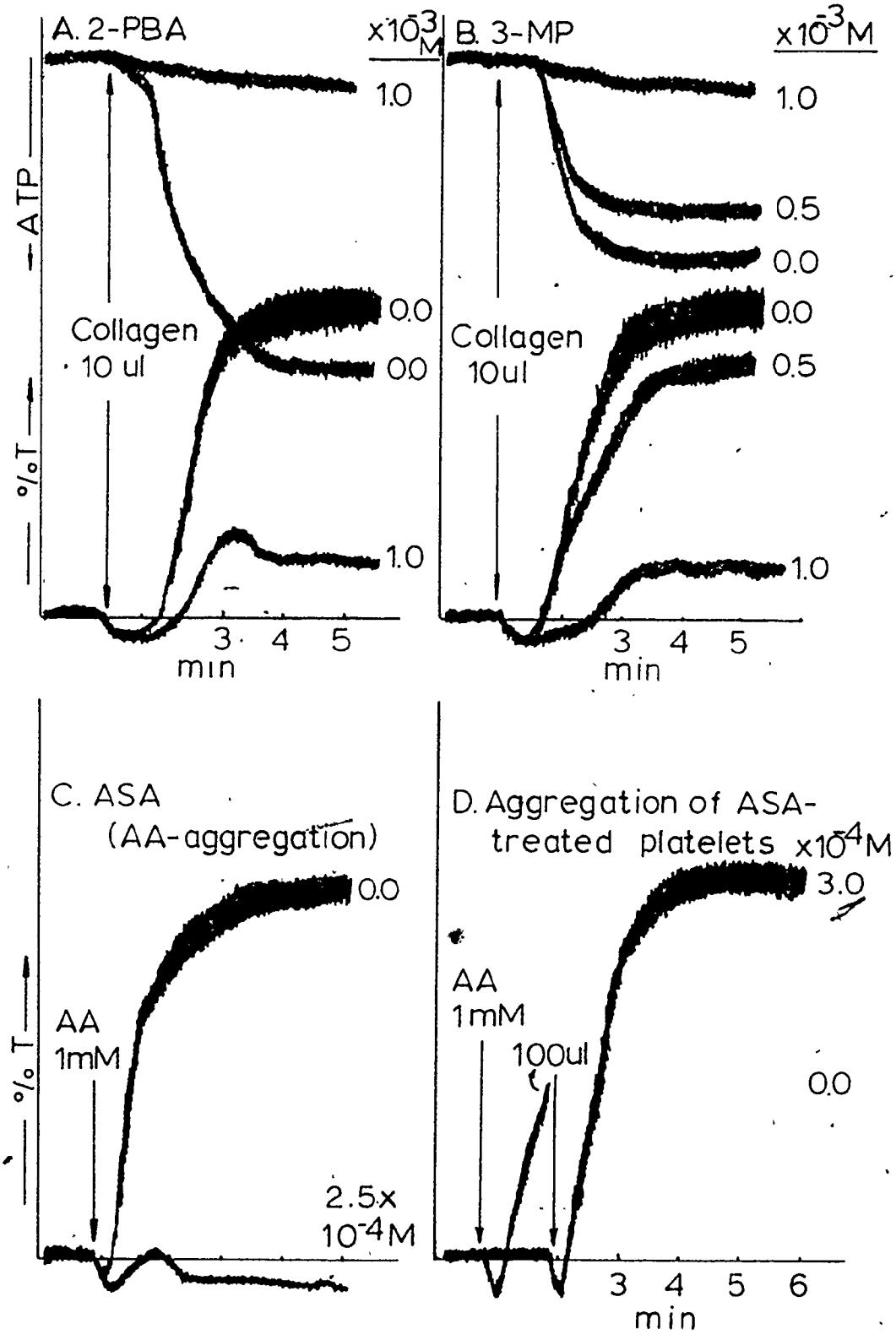

A. 2-PBA
x10⁻³M
1.0
0.0
0.0
1.0
Collagen
10 ul
-ATP
%T
3 4 5
min
B. 3-MP
x10⁻³M
1.0
0.5
0.0
0.0
0.5
1.0
Collagen
10ul
3 4 5
min
C. ASA
(AA-aggregation)
0.0
AA
1mM
2.5x
10⁻⁴M
%T
D. Aggregation of ASA-
treated platelets x10⁻⁴M
3.0
AA
1mM
100ul
0.0
3 4 5 6
min

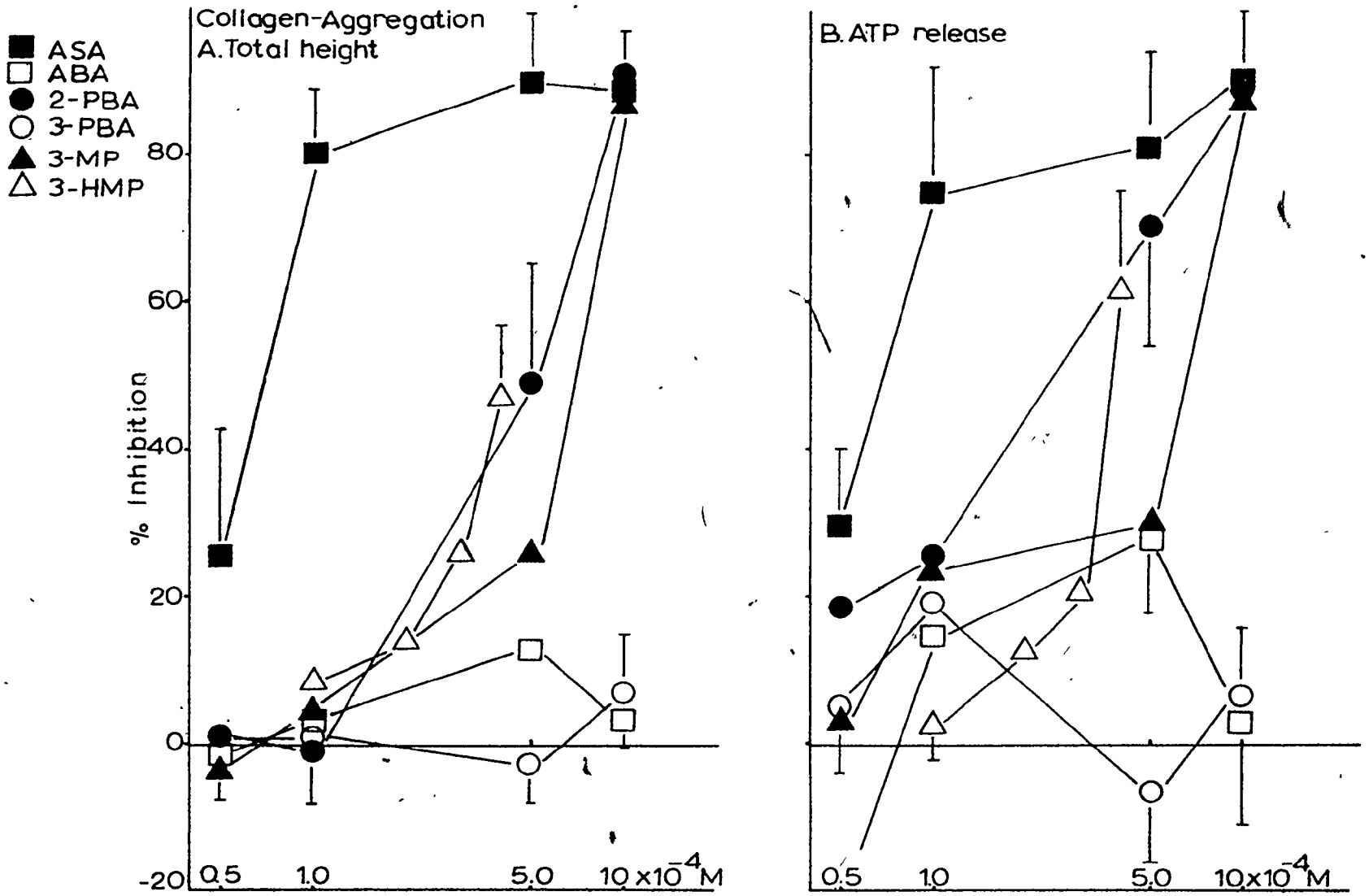
Fig. 6 Dose-response effects of benzoic acid analogs on collagen-induced aggregation and the simultaneous release of ATP.

A. 5 min total height of aggregation curves

B. ATP release

Drugs were preincubated with platelets for 5 min before the addition of collagen.
% Inhibition of release of ATP by ABA at 5×10^{-4} M was -23 ± 18.

For each point n=4.


ASA
ABA
2-PBA
3-PBA
3-MP
3-HMP
Collagen-Aggregation
A. Total height
% Inhibition
80
60
40
20
0
-20
0.5
1.0
5.0
10 x10⁻⁴M
B. ATP release
0.5
1.0
5.0
10x10⁻⁴M

agents were incubated in PRP for 5 minutes prior to addition of the aggregating agent. Based on the report by Levy and Leonards (1966) that an oral dose of 2 to 4 360 mg ASA tablets in human adults resulted in a blood concentration of 0.5 mM, drug concentrations in this study were set in this range. Although ASA had no effect on the first phase of ADP-induced aggregation at these concentrations, it was the most potent inhibitor of second phase aggregation and the release of ATP. 2-PBA demonstrated the same qualitative properties at a lower potency and it too was a potent inhibitor of the release of ATP. The aggregation results are in agreement with the report of Mills et al (1974). The close relationship of second phase aggregation to release of ATP is demonstrated here. Mills et al (1974) reported a potentiation of second phase platelet aggregation when 3-PBA was tested on PRP without preincubation and, here, 3-PBA caused a slight potentiation of ADP-aggregation but, only at lower doses. An increase in release of ATP was measured at 10^{-4} M final concentration. The small degree of potentiation of aggregation seen here compared to the previous report may be accounted for by possible physical interference caused by the firefly extract, but the results are qualitatively confirming. ABA had little effect on platelet aggregation and release except, perhaps, at the higher concentrations while 3-MP, designed to resemble the ring form of ABA, inhibited both phases of platelet aggregation in a dose-response manner. It was a less potent inhibitor of release than ASA despite the inhibition of both phases of ADP-induced aggregation. This is further evidence that these agents, with close structural similarities to ASA, inhibit platelet aggregation through different mechanisms.

3HMP was first discovered as a pharmacologically active agent by

Cerskus (1978). This agent was the most potent inhibitor of total ADP-induced platelet aggregation used in this study but, despite this fact, it was less potent than ASA in inhibiting second phase aggregation and release. Porter et al (1976) studied the platelet inhibitory activity of a series of monocyclic peroxides. Some of these agents not only inhibited first and second phase aggregation, but also aggregation induced by PGH_2. The mechanism of inhibition was not known. While ASA demonstrated an extremely steep dose-response effect for the inhibition of second phase aggregation 3HMP exhibited a steep dose-response curve on total platelet aggregation. The inhibition of first phase and total aggregation using 3-MP and 3HMP occurred over the same dose range as the inhibition of release and this may point to a more general mechanism for the inhibition of platelet aggregation rather than a specific mechanism for inhibiting second phase and release and another mechanism for inhibiting first phase aggregation. The effects of these agents on ADP aggregation are similar to those reported for VK774, a compound with potent PDE inhibiting activity (Philp et al 1974, McElroy and Philp 1975) (Fig. 2B).

The effects of these agents on low dose collagen aggregation generally reflect the same profile of activity seen on the second phase of ADP platelet aggregation. The dependency of collagen-induced platelet aggregation on the arachidonate pathway is demonstrated by the potency of ASA, which is an irreversible cyclo-oxygenase enzyme inhibitor. The inhibition of the release of ATP followed the inhibition of collagen aggregation.

Further information concerning the mechanism of action of these agents may be obtained through a study of aggregation and release

induced by a much higher dose of collagen. Kinlough-Rathbone et al (1980) reported on the weak effect that the CP/CPK system (which removes ADP) in combination with a NSAID had on this type of aggregation and this may allow a further separation of the activities of 3-MP and 3HMP versus ASA.

In these studies the release of ATP always followed the second phase of ADP aggregation or collagen aggregation and the inhibition pattern for one was similar for the other. If these are separate but parallel events (Charo et al 1977a,1977b) they are closely associated by some mechanism. First phase ADP aggregation is separable from ATP release.

A brief study was designed to assay the effects of these agents on AA-induced platelet aggregation. ASA appeared to be the most potent inhibitor although 3HMP was used at a lower concentration than the other agents. 3-MP was clearly less potent than 2-PBA and ASA and also 3HMP and these results reflect the effects of these agents on collagen aggregation and release.

The effects of the NSAID on ADP, collagen and AA-induced platelet function as measured in the platelet aggregometer are dramatic. The design of an in vitro technique which will adequately reflect the in vivo situation is essential in the study of antithrombotic activity of drugs. Fig. 5D demonstrates, for instance, that platelets stimulated with arachidonic acid rapidly produce proaggregatory substances which can overcome the inhibitory effects of ASA on platelets as originally reported by Hamberg et al (1974). This is especially important in light of the report that small numbers of functional platelets in a population of ASA treated platelets can overcome the inhibitory effect of ASA

(Cerskus et al 1980). Systems such as these (see also Kinlough-Rathbone et al 1980) may prove more valuable in understanding the important controlling mechanisms in thrombus formation.

In this study there was a direct relation between the inhibition of ADP second phase platelet aggregation or low dose collagen aggregation and the inhibition of the release reaction as reflected in the release of ATP. None of the agents tested caused a specific inhibition of either of these types of platelet aggregation or the release reaction.

2.3.2 Effects of Benzoic Acid Analogs on Platelet Prostaglandin Synthesis

2.3.2.1 Introduction

Prostaglandins (PG) are not stored and their presence indicates new synthesis from esterified precursors (Lands and Samuelsson 1968). Arachidonic acid (AA) (20:4) (20 carbon fatty acid with 4 double bonds), the precursor of the predominant types of PGs is formed by elongation and desaturation of linoleate (18:2). The highest concentration of AA is in phospholipids, although the highest total amount may be in the triglycerides (Lands 1979). Bills et al (1976) reported the highly specific release of AA (compared to other fatty acids) from platelets by platelet agonists, however, their report of a phospholipase A_2 enzyme mediating this release has been difficult to demonstrate. The presence of a phospholipase C and a diglyceride lipase, which, in combination, mediate the release of AA has been suggested (Bell et al 1980; Gerrard and Graff 1980) while Lapetina et al (1981) have suggested that phospholipase A_2 may be involved, but indirectly, in a phosphatidate/lysophosphatidate-mediated multistep mechanism. The AA of membrane phosphatidylcholine and phosphatidylinositol is rapidly labelled with ^{14}C-AA and AA from these stores has been shown to be preferentially liberated upon thrombin stimulation (Bills et al 1976). Rittenhouse-Simmons et al (1977) suggested that the primary source of AA for PG synthesis may be the phosphatidylethanolamine plasmalogen fraction which becomes enriched in AA after thrombin stimulation.

Proteolytic action may be needed for the activation of phospholipase activity (Feinstein et al 1977), and cAMP inhibits the release of AA (Gerrard et al 1977, Lapetina et al 1977).

Upon release, substrate acids may bind to proteins, become esterified to lipid, become oxygenated by lipoxygenases or experience competition at the PG synthesizing enzymes instead of being converted to PGs (Lands 1979). Because nonesterified acids are substrates and hydroperoxides serve as cyclo-oxygenase activators, neither of which are found in appreciable amounts in the cell, normal physiological PG levels are low (Lands 1979). The PG endoperoxide synthetase enzyme (EC 1.14.99.1) or cyclo-oxygenase, inserts oxygen into the polyunsaturated fatty acid and rearranges the acyl chain to form the various products. This membrane-bound glycoprotein is similar in most systems (Smith and Wilken 1977) and consists of 2 identical polypeptide chains, one heme and is devoid of nonheme iron (Van der Ouderaa et al 1980). At least 5 uM O_2 is required for appreciable activity (Lands et al 1978). Peroxidase activity involved in the conversion of PGG_2 to PGH_2 appears connected to the main endoperoxide forming enzyme (Miyamoto et al 1976).

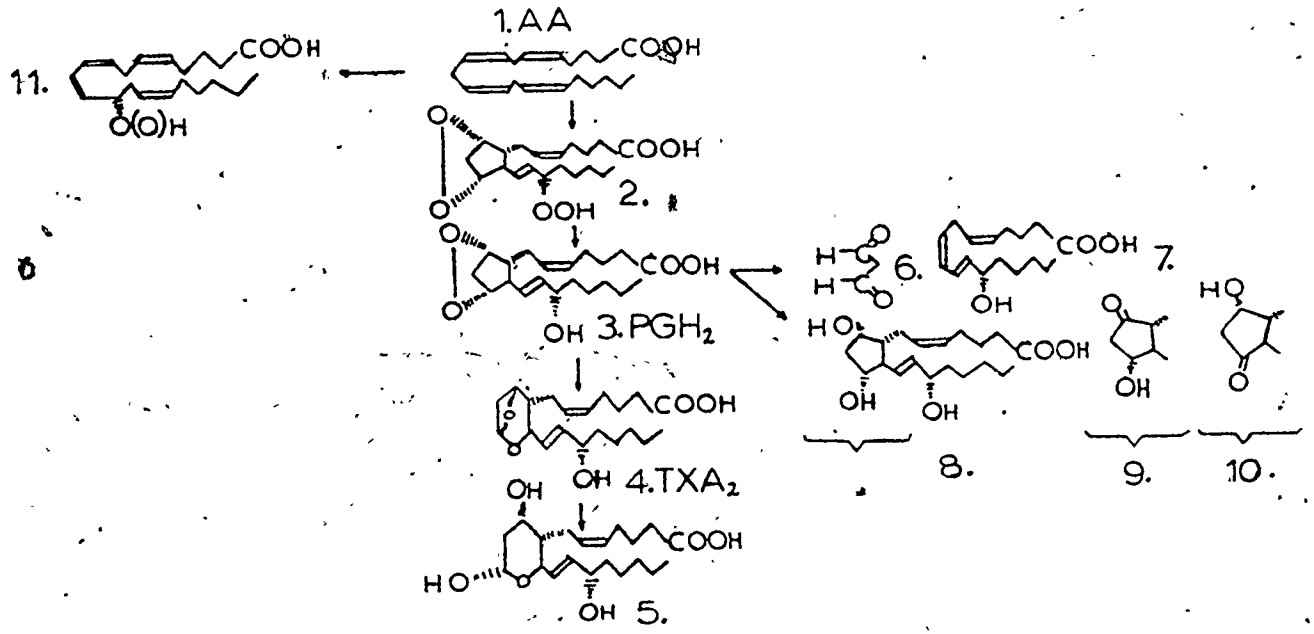
Cook and Lands (1975) reported a positive feedback in cyclo-oxygenase activity and small amounts of hydroperoxides were shown to trigger the synthesis and to allow PG synthesis to continue (Lands 1979). Other agents including ascorbic acid, adrenaline and tryptophan also activate the activity and Peterson et al (1980a, 1980b) suggested that these agents, including the hydroperoxides, reduced ferric heme to the ferrous state, which is necessary for enzyme activity. Cyclo-oxygenase studies have demonstrated a progressive inactivation of the enzyme as the reaction proceeds (Smith and Lands 1972) and Egan et

al (1976) suggested that this occurred through a free radical mechanism. Both the cyclo-oxygenase and peroxidase activities decreased after 15-30 seconds of catalysis (Miyamoto et al 1976) and both this and the hydrogen peroxide (H_2O_2) destruction of the enzyme were prevented by hydroquinone (Van der Ouderaa et al 1977). This suggests that the inactivator is a hydroperoxy derivative of the substrate fatty acid. Hemler and Lands (1980) suggested that the enzyme caused the formation of fatty acid radicals which react with oxygen and that the self-catalyzed enzyme destruction was a separate feature intrinsic to cyclo-oxygenase catalysis and probably mediated by reaction intermediates.

In platelets, roughly equal amounts of thromboxane B_2 (TXB_2), 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde (MDA) as well as the lipoxygenase product 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) are formed but the ratio of TXB_2 to other PGs is much higher (Pace-Asciak 1977) (Fig. 7). Thromboxane synthetase has been solubilized and resolved from cyclo-oxygenase and it converts PGH_2 to TXA_2 (Yoshimoto et al 1977). The enzyme is found in the particulate fraction associated with the dense tubular system (Hammarstrom and Falardeau 1977). Thromboxane synthetase catalyzed the formation of HHT and MDA (Hammarstrom and Diczfalusy 1980) and at low levels of AA, the amount of MDA produced equalled the amount of TXB_2 produced, while at higher concentrations of AA, the MDA levels were higher due to nonspecific oxidation of AA (Best et al 1980). Thromboxane synthetase uses PGH_2 as the substrate for thromboxane production, but under certain conditions will convert PGG_2 to 15-hydroperoxy thromboxane A_2 and

Fig. 7 The major cyclo-oxygenase and lipoxygenase products of platelet AA metabolism.

1. Arachidonic acid (AA)
2. Prostaglandin G_2 (PGG_2)
3. PGH_2
4. Thromboxane A_2 (TXA_2)
5. TXB_2
6. Malondialdehyde (MDA)
7. 12-hydroxyheptadecatrienoic acid (HHT)
8. $PGF_{2\alpha}$
9. PGE_2
10. PGD_2
11. 12-hydroxy (hydroperoxy) eicosatetraenoic acid (12-H(P)ETE)


1. AA
11.
COOH
O(O)H
OOH
2.
OH
3. PGH2
OH
4. TXA2
HO
5.
6.
7.
8.
9.
10.

12-hydroperoxy-5,8,10-heptadecatrienoic acid (Hammarstrom 1980). Although the half-life of TXA_2 is only 40 seconds, it may exist longer in plasma due to albumin stabilization (Folco et al 1977).

When PGH_2 is dissolved in aqueous solution, it spontaneously decomposes at 37° C to PGE_2 and PGD_2 (Smith et al 1980) and the endoperoxides are extremely labile in albumin solutions, decomposing to PGD_2 (Hamberg and Fredholm 1976). The release of PG endoperoxides by aggregating platelets has been reported (Hamberg et al 1974c, Smith et al 1974a) and these endoperoxides likely decompose to PGD_2. Oelz et al (1977) reported the production of small amounts of PGD_2 from thrombin stimulated platelets, however, Needleman et al (1979) reported opposing results. He claimed that intrinsically generated endoperoxide is normally so efficiently coupled to thromboxane synthetase that little PGD_2 or PGE_2 formation occurs. In the presence of an inhibitor of thromboxane synthetase however, PG endoperoxides were produced and released. The matter is still controversial, especially in light of the stability of PGD_2, which would allow it to accumulate and serve as a feedback inhibitor of thrombus formation (Smith et al 1974a, 1974b).

Another route of AA metabolism in platelets is through the lipoxygenase enzyme (Nugteren 1975). This enzyme has been found in the soluble cytoplasmic fraction of platelet homogenates although a membrane enzyme has also been reported (Sun et al 1980). This enzyme is not inhibited by indomethacin and both this enzyme and the cyclo-oxygenase enzyme have been reported to produce covalently binding reactive intermediates (Wilson et al 1979). The unsaturated hydroperoxides produced by this pathway may serve to stimulate the rate of PG biosynthesis (Lands 1979) or inhibit it (Aharony et al 1981).

The mechanism by which thromboxane and endoperoxides cause platelet aggregation remains to be determined. A model has been constructed by White and associates (1978) and here the movement of calcium from stores in the dense tubular system is the final common pathway for platelet activation as follows: 1. receptor activation occurs 2. this leads to activation of an AA-releasing mechanism by small amounts of calcium 3. PG production occurs in the membrane tubules (White and Gerrard 1978), however, if the cyclo-oxygenase is blocked by an ASA-like drug, if the related peroxidase is blocked by aminotriazole or if AA is prevented from transforming to a free radical by vitamin E or nitroblue tetrazolium (White et al 1977), this PG production is blocked 4. TXA_2 and the endoperoxides transport Ca^{++} to the contractile elements where it is released by Mg^{++} exchange or hydrolysis 5. the endoperoxide-induced reduction of cAMP (Gorman et al 1977b) allows the accumulation of Ca^{++} in the cytoplasm 6. calcium levels can be reduced and maintained by stimulation of the cAMP dependent calcium extrusion pump ATPase. Pseudopod formation may be a separate event from the initial calcium activation (White and Gerrard 1978). In line with this model, Kaisar-Glanzmann et al (1977) reported the membrane vesicles derived from platelets concentrate calcium and that the effect was stimulated by cAMP in the presence of ATP. Cyclic AMP has been reported to block the cyclo-oxygenase enzyme (Malmsten et al 1976) and PGG_2 aggregation (Salzman 1977). Calcium inhibits adenylate cyclase activity (Rodan and Feinstein 1976) and the endoperoxide-thromboxane system is able to counteract the stimulation of adenylate cyclase in platelets (Gorman et al 1977a). Johnson et al (1980) reported the transformation of AA-sensitive dog platelets to AA-resistant platelets through agents

that increase cAMP and the synergism between adrenaline and other aggregating agents has been suggested to be through its cAMP lowering effect (Patscheke 1980). Morphological effects of endoperoxides and thromboxanes resemble the effects of calcium ionophores (White and Gerrard 1978) and phospholipids themselves, may serve as ionophores (Ikeda et al 1979). Furthermore the platelet inhibiting PGs PGE_1, PGD_2 and PGI_2 exert at least part of their inhibiting effect through stimulation of adenylate cyclase (Gorman et al 1977a, Di Minno et al 1981). Further evidence supporting this model has been summarized by Philp (1981).

There is a rapid loss of PG activity through enzymatic oxidation of the 15-hydroxyl group by 15-hydroxydehydrogenase. Other routes of PG metabolism include 13-reductase activity and beta and omega oxidation (Lands 1979).

To understand the role of PG synthesis in platelet function, areas requiring further study involve the question of whether PGH_2 must be converted to TXA_2 for aggregation to take place, the role of PGD_2 in platelet aggregation and thrombosis (mentioned previously) and the role of the products of the lipoxygenase pathway in platelet function (Dutilh et al 1980).

Prostaglandin and thromboxane synthesis may be inhibited by a number of mechanisms other than inhibition of the cyclo-oxygenase enzyme. Anti-inflammatory steroids have been shown to inhibit the release of substrate (Blackwell et al 1978), reduce the transport of nonesterified AA to cyclo-oxygenase (Turner et al 1975) and block the efflux of PG from the site of synthesis by blockade of carrier-mediated

release (Chang et al 1977). A number of thromboxane synthetase inhibitors such as L-8027, dipyridamole, imidazole, azo and epoxy analogs of PGH_2 (Lands 1979), nordihydroguaiaretic acid and 12-HPETE (Samuelsson et al 1978) have been reported. Because four types of ligands are necessary for cyclo-oxygenase activity, namely heme, oxygen, hydroperoxides and substrate, a variety of mechanisms of inhibition are possible. The unusual kinetics of the enzyme complicate enzyme inhibition studies. Free radical scavenging substances have a dual effect on the enzyme (Egan et al 1980). Stimulation at lower concentrations have been suggested to reflect enzyme preservation from free radical destruction and inhibition at higher concentrations may be due to interference with the hydroperoxide activator. Phenolic compounds, which are free radical scavengers, may lower peroxide tone, serve as free radical traps, serve as oxidizable substrates for the enzyme peroxidases and also competitively inhibit PG cyclo-oxygenase (Dewhirst 1980). Flavenoid compounds can also activate the cyclo-oxygenase and inhibit the lipoxygenase and/or cyclo-oxygenase enzymes (Baumeny et al 1980). Acetaminophen competes reversibly for both the substrate and peroxide activator sites (Lands et al 1976) and differences in concentration of substrate or peroxide were reported to account for the paradoxical reports of the effect of this agent on the cyclo-oxygenase enzymes of various tissues.

The inhibition of PG endoperoxide synthetase has been reviewed by Flower (1974), Gryglewski (1974) and recently by Shen (1979). Various templates have been suggested for the design of agents which will specifically inhibit PG synthesis. The models of Scherrer (1974) and Gund and Shen (1977) are based on the assumption that the carboxyl

function of the NSAID binds to the enzyme normally occupied by the carboxyl group of the fatty acid precursor. While the Scherrer model accomodates ASA, the model of Gund and Shen does not and, based on the partial inhibition of ASA enzyme acetylation by indomethacin, they proposed two inhibitor receptor sites. Parantainen (1979) proposed that NSAID bound to the coenzyme site of the cyclo-oxygenase. Appleton and Brown (1979) proposed a NSAID template design based on the conformation of the peroxy radical precursor of PGG_2 existing immediately prior to cyclization to PGs. This model appears to better accomodate more NSAID but it is unclear if and where ASA would bind. Based on reports that ibuprofen inhibits ASA-acetylation of the cyclo-oxygenase enzyme (Parks et al 1981b) it is likely that ASA binds to the same site as other NSAID. Humes et al (1981) have provided data indicating at least two sites on the cyclo-oxygenase enzyme: a substrate binding catalytic site and a supplementary binding site to which the NSAID show particularly high affinity.

The majority of the ASA-like drugs appear to inhibit the enzyme in a "competitive-irreversible" way (Flower 1974) with the inhibitor combining in an irreversible, time dependent manner, however, the initial degree of inhibition depends on the substrate as the presence of the substrate at this site reduces the velocity of inhibitor-enzyme combination. An alternative explanation is that the inhibitor binds to another site which is in sufficient proximity to the catalytic site to reduce its affinity for the substrate. Raz et al (1973) reported that indomethacin was, like ASA, an irreversible inhibitor of sheep seminal vesicle enzyme. Ali and McDonald (1978) reported that indomethacin, ASA and naproxen caused time-dependent irreversible inhibition of the

platelet cyclo-oxygenase whereas phenylbutazone, sulfinpyrazone and fenoprofen did not. In spite of this fact, indomethacin inhibits PG synthesis in vivo only as long as the drug is present in sufficient concentration and the reasons for this are, as yet, unknown. The irreversible inactivation appears dependent on the carboxylic acid group (Rome et al 1975).

According to Roth and Majerus (1975) ASA binds to the substrate site and, in addition, provides a progressive irreversible inactivation of the PG cyclo-oxygenase enzyme that is associated with, and may actually precede, acetylation of subunits (Rome et al 1976). Acetylation occurs at a serine hydroxyl and this is reported to be other than an N-terminal group (Van der Ouderaa et al 1980). Although salicylic acid was much less active than ASA in vitro, 5-(4-flurophenyl) and 5-(2'-4-diflurophenyl) salicylic acids were potent PG synthesis inhibitors (Shen et al 1974). The 5-cyclohexyl , 5-phenoxy and 4-phenyl analogs of salicylic acid were much less active but this study demonstrated that the o-acetyl group was not necessary for antiPG synthesis activity. Agents with benzyloxy groups in the 2 or 4 position on the benzoic acid also had reduced potency compared to ASA (Gryglewski 1974).

The optimal enzyme acetylation by ASA seems dependent on the hemoprotein activators that give the optimal enzyme oxygenase activity (Shen 1979). The acetylation can be inhibited by thrombin stimulation as well as AA stimulation of platelets (Minkes et al 1977) and salicylate both inhibited and reversed the inhibitory effect on rat platelet MDA production (Merino et al 1980).

Salicylates may affect AA metabolism in other ways including

inhibition of the release of fatty acids (Pilo and Raz 1981) and inhibition of the conversion of HPETE to HETE (Siegel et al 1979b).

Cerskus (1978) studied the present series of benzoic acid analogs and found that substitution of a 2-propionyloxy group (2-PBA) for the acetoxy group resulted in PG synthesis inhibition but at a reduced potency. Movement of this group to the 3-position (3-PBA) led to a slight increase in enzyme activity. Changing the acetoxy group to an acetyl group (ABA) resulted in a loss of activity, however, 3-MP, designed to resemble the ring tautomer of ABA, was a weak inhibitor. This compound photo-oxidized to a hydroperoxide compound, 3HMP, which surprisingly, was more potent than ASA in inhibiting PG synthesis despite the weak activity of 3-MP.

Imidazole is an inhibitor of thromboxane synthetase and is relatively specific in this regard (Moncada et al 1977, Needleman et al 1977). When imidazole is incubated with intact platelets and ^{14}C-AA, the lone large TLC peak of radioactivity corresponding to TXB_2 in control platelets disappears and a major peak at PGE_2 accompanied by two smaller peaks at PGD_2 and $PGF_{2\alpha}$ results (Needleman et al 1979).

Ali et al (1977) reported that freeze thawed washed platelets produced equal amounts of PGD_2 and TXB_2 in contrast to intact platelets and these were both inhibited by sulfinpyrazone, phenylbutazone, indomethacin, ASA and the cyclo-oxygenase and lipoxygenase inhibitor eicosatetraynoic acid (ETYA).

The purpose of this study was to confirm the work of Cerskus (1978) of the effects of the benzoic acid analogs on the total PG synthesis from labelled AA by freeze thawed washed platelets and then to examine the effects of these agents on the TLC profile of PG synthesis by this

preparation to test for specific effects of these agents on the different PGs produced. If the formation of PGD_2 from PG endoperoxides is independent of thromboxane synthesis, these peaks should be affected differentially by a specific thromboxane synthetase inhibitor. Through differential effects on the PGD_2 and TXA_2 peaks, agents such as ABA, 3-PBA or 3-MP may exert their effects and yet not show a change in total PG production.

2.3.2.2 **Methods**

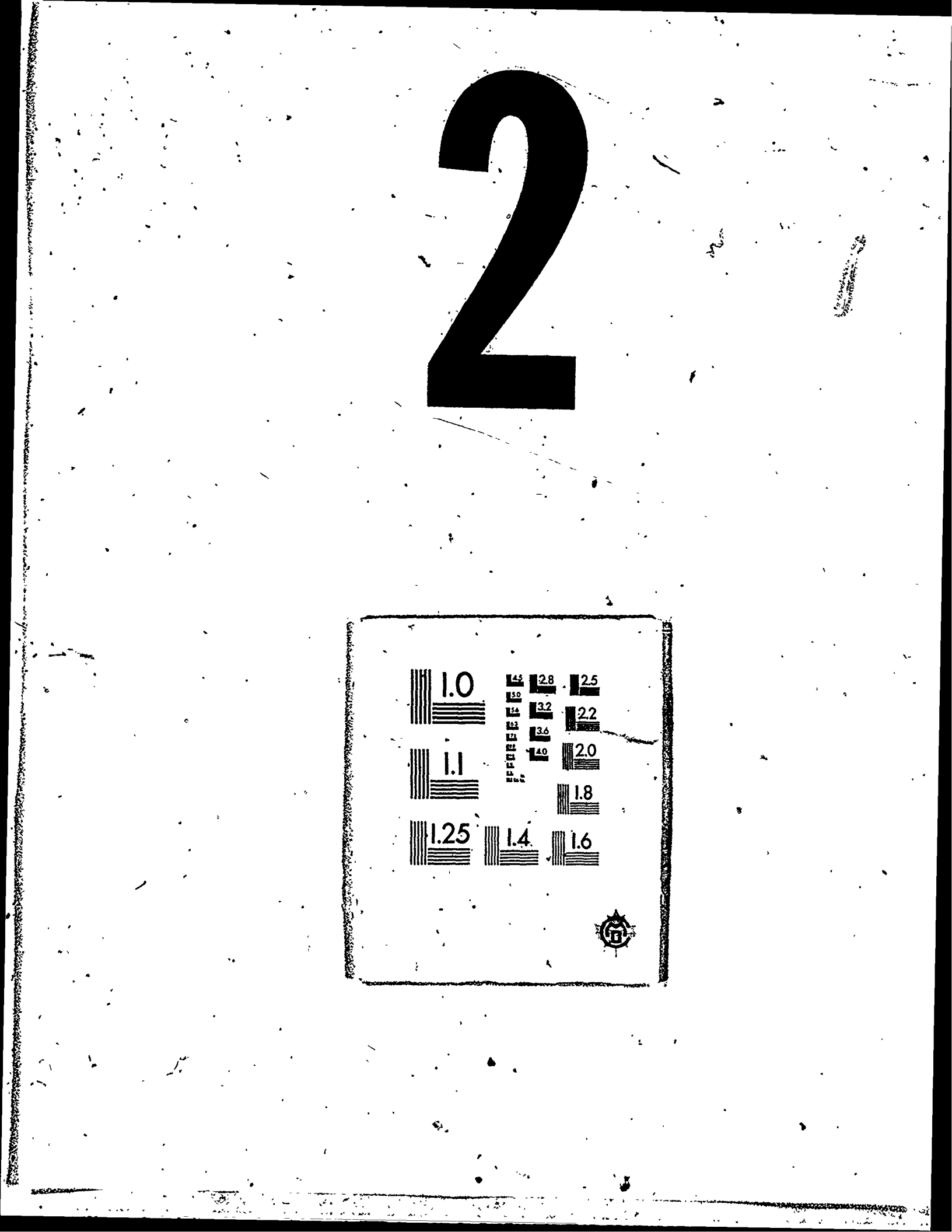
Platelet PG Synthesis

Preparation of freeze thawed washed platelet lysates

PRP was prepared as described previously and to this was added 136mM disodium ethylenediamine tetraacetate (EDTA, Fisher Scientific Co., USA) to a final concentration of 5mM. The PRP was spun for 20 minutes at 967 x g and the supernatant plasma removed leaving a platelet pellet. The pellet was resuspended in 0.14M saline containing 0.015M EDTA (pH 6.3) to a quarter of the original volume using a Pasteur pipette. The resuspended platelets were again centrifuged at 967 x g for 20 minutes, the supernatant removed and the platelets resuspended in 0.05M Tris-HCl buffer containing 0.015M EDTA (pH 7.4) to a quarter of the PRP volume and a platelet count was done at this stage. The final volume was determined such that the platelet count was $1x10^9$ platelets/ml. The suspension was placed into Eppendorf Microtest tubes (Fisher Scientific Co., Toronto) in 250ul portions and frozen at -70° C until used. Washed platelet lysates were prepared by thawing the washed platelets at 37° C followed by rapid freezing at -70° C for a total of three freeze thaws. In some studies washed, intact platelets, prepared that day, were used.

Incubation and extraction procedure

PG synthesis was studied according to the method of Ali et al. (1977) with modifications as described below. Samples of freeze thawed platelet lysates were incubated at 37° C for 7 minutes in the presence of 50ul Tris-HCl buffer (0.05M pH 7.4) or 50ul drug made up in Tris-HCl buffer. ^{14}C-AA (54.6mCi/mmol, Amersham Searle, Oakville, Ontario) was


2
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0

mixed with nonlabelled AA (Gr.I, Sigma Chemical Co., St. Louis) and dried under nitrogen. The AA was dissolved in 10-20ul ethanol, depending on the final volume, and diluted with Tris buffer (pH 7.4) to produce final concentrations of 5, 10uM and other concentrations with specific activities of usually 10µCi/umol but 5uCi/umol for the higher concentrations. Two hundred ul AA were added to the lysates and after 1 minute the reaction was terminated by transferring the lysates to tubes containing 2.25 ml absolute ethanol and 1.25 ml saline in 16 x 125mm glass tubes. All additions were made using Oxford Samplers. The solutions were acidified to a pH of 3.0 with 9.2% formic acid (Fisher Scientific Co., USA) and the mixture was extracted with 6.25 ml chloroform by vortexing for 30 seconds. The contents of the tube were transferred to a 15 ml centrifuge tube and centrifuged at 967 x g for 3 minutes to facilitate the separation of the two phases. The upper aqueous phase, together with precipitated protein were removed and the lower organic phase was evaporated to dryness under a stream of nitrogen.

The dried extracts were redissolved in 500ul 2% methanol in chloroform ($MeOH/CHCl_3$) by vortexing for 30 seconds and the PGs and thromboxane fraction was separated from other products and unused substrate by silicic acid column chromatography. Activated silicic acid (heated at 125° C for approximately 1.5 hours) (0.6g per column, 60-200 mesh, Sigma Chemical Co., St. Louis) was suspended in 6% $MeOH/CHCl_3$ (3 ml/column) and poured into glass wool plugged columns (0.4 x 12.5cm) to a height of 11cm. The columns were washed with 2 ml 6% $MeOH/CHCl_3$ followed by 5 ml 2% $MeOH/CHCl_3$. The samples were applied to the columns and 6 ml of 2% $MeOH/CHCl_3$ were added to remove unused AA and

lipoxygenase products. The PG products were next eluted with 6 ml of 6% MeOH/$CHCl_3$. In some experiments this fraction was dried under air, redissolved with 500ul methanol and mixed with 10 ml liquid scintillation counting fluid in 20 ml plastic scintillation vials (Amersham Searle, Oakville, Ontario). Samples were counted using a Searle Diagnostic Liquid Scintillation counter. In other experiments, the 6% MeOH/$CHCl_3$ fraction was dried under nitrogen, redissolved in 10ul ethanol and spotted on silica gel G plates (100 u thick, Eastman Chromogram Plates, Fisher Scientific Co., Toronto) activated at 100° C for 15-30 minutes. On the same spot 2ul samples of standards of PGD_2, PGE_2, TXB_2, $PGF_{2\alpha}$-tromethamine salt and PGI_2 sodium salt (Upjohn Co., Kalamazoo, Michigan) dissolved in ethanol in a concentration of 1 mg/ml, were placed as markers. The plates were developed twice under nonsaturating conditions in the organic phase of ethyl acetate/acetic acid/isooctane/water (110:20:50:100 v:v) unless otherwise stated. Standards were stained in iodine vapour, the plate was cut up, and the pieces placed in scintillation vials. Methanol (500ul) was added and this was mixed with 10 ml of scintillation cocktail and counted.

The small amount of plate material in each vial did not quench the scintillation counts significantly and hence all data was presented as counts per minute (CPM). The control % conversion to PGs was 2%.

^{14}C-AA was stored at -20° C in a nitrogen atmosphere in toluene. Nonlabelled AA was made up in a 1 mg/ml solution in ethanol and stored under nitrogen at -20° C. The scintillation cocktail was made up of 12g PPO (Amersham) and 1.2g POPOP (Amersham) dissolved in scintillation grade toluene, 2400 ml. All organic solvents were of reagent grade purity from Fisher Scientific Co., Toronto (see Appendix IV).

2.3.2.3 **Results**

The effect of ASA, 2-PBA and 3HMP at 100uM final concentration on the production of PGs from various concentrations of ^{14}C-AA are shown in Fig. 8. This represents total PG as eluted in the 6% MeOH/$CHCl_3$ from silicic acid column chromatography. The time dependent nature of ASA inhibition is shown by the increased inhibition at 7 compared to 0 minutes drug preincubation (Fig. 8A). 2-PBA and 3HMP were also potent inhibitors after 7 minutes preincubation (Fig. 8B,C).

All agents were tested on total PG synthesis by freeze thawed platelet lysates. The AA concentration was set at 10uM based on preliminary studies demonstrating good PGD_2 and TXB_2 production at this concentration (Fig. 9). 2-PBA was less active than ASA but retained potent antiPG synthesis effects. 3-PBA and ABA, in contrast, did not demonstrate any inhibitory activity. 3-MP showed a slight trend towards inhibition but only at the highest dose while 3HMP, the hydroperoxy analog of 3-MP, was the most potent inhibitor - even compared to ASA. Agents demonstrating activity at 10uM final AA concentration were also tested at 5uM AA and the same profile of inhibition was evident.

Separation of the AA metabolic products in the 6% MeOH/$CHCl_3$ fraction resulted in 2 peaks of activity corresponding to PGD_2 and TXB_2 (Fig. 10). Imidazole (5mM) was used as an example of a thromboxane synthetase inhibitor (Fig. 10) and it inhibited the thromboxane peak, partially inhibited the PGD_2 peak and led to an increase in the peak corresponding to PGE_2. All agents previously demonstrating inhibitory activity were used at their approximate ID_{50} concentrations (the concentration causing a 50% reduction in enzyme activity). All other

Fig. 8 The effects of A. ASA B. 2-PBA C. 3HMP on freeze thawed platelet lysate conversion of ^{14}C-AA to PGs.

Benzoic acid analogs were preincubated with platelet lysates for 7 min afterwhich ^{14}C-AA was added and the reaction run for 1 min.
ASA was also tested without a preincubation period.
Radioactivity in the 6% MeOH/$CHCl_3$ fraction from silicic acid column chromatography represents PG and TX products formed from ^{14}C-AA.
Each point is the average of duplicate determinations.

nM AA converted to PG=

$$\frac{\text{CPM in 6\% MeOH/CHCl}_3 - \text{background}}{\text{Total CPM} - \text{background}} \times \text{nanomoles added}$$

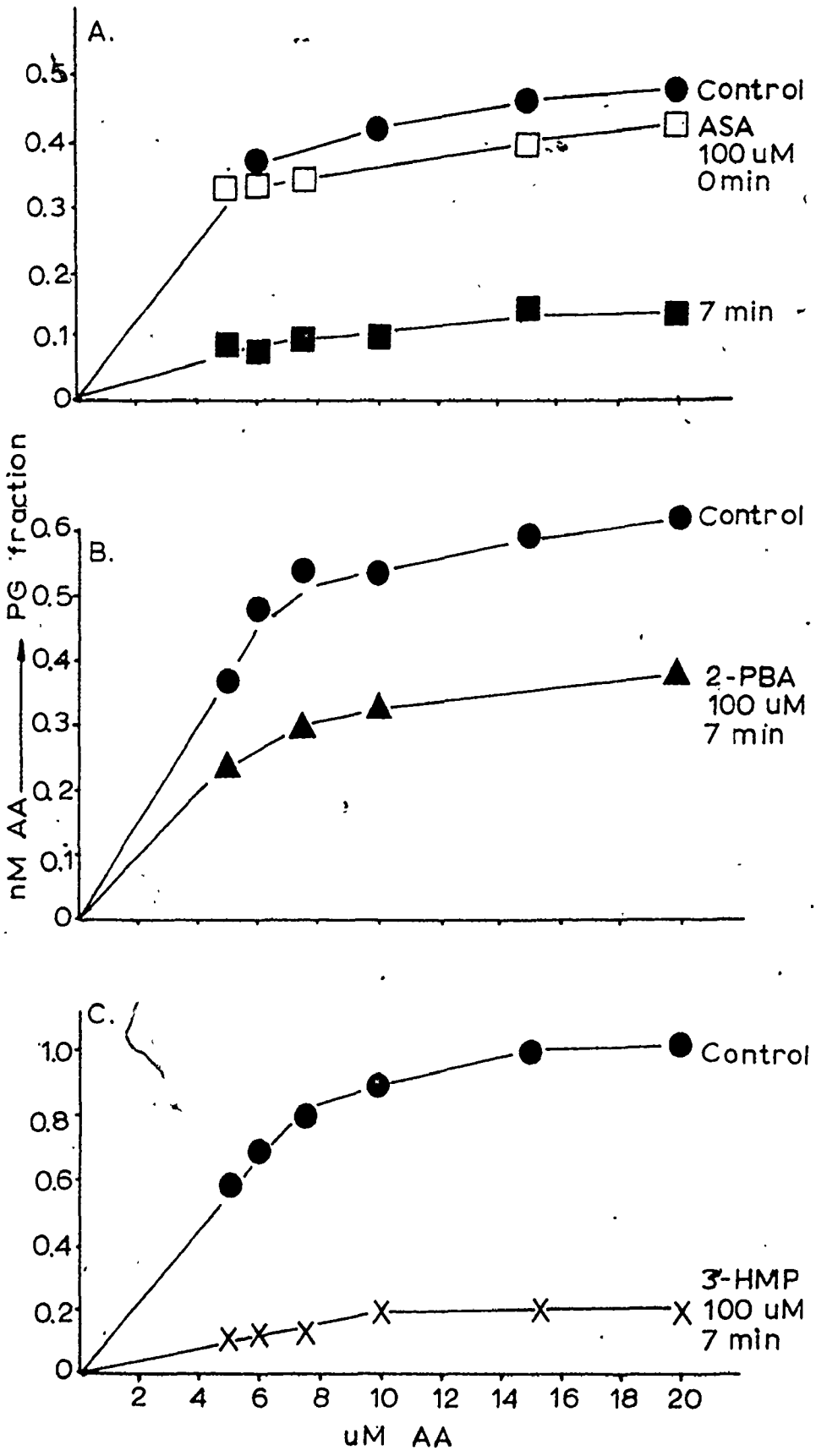

A.
0.5
0.4
0.3
0.2
0.1
0
Control
ASA
100 uM
0 min
7 min
B.
PG fraction
nM AA
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
2-PBA
100 uM
7 min
C.
1.0
0.8
0.6
0.4
0.2
0
Control
3-HMP
100 uM
7 min
2 4 6 8 10 12 14 16 18 20
uM AA

Fig. 9 Dose-response effects of benzoic acid analogs on total PG production by platelet lysates from A. 10 uM ^{14}C-AA and B. 5 uM ^{14}C-AA.

Benzoic acid analogs were preincubated with platelet lysates for 7 min afterwhich ^{14}C-AA was added and the reaction run for 1 min. Radioactivity in the 6% MeOH/$CHCl_3$ fraction from silicic acid column chromatography of incubate extracts represents PG and TX products. % Inhibition was calculated by comparison with controls in which buffer was added instead of benzoic acids.
Each line in A. and B. was drawn by visual estimation from three points representing the average of duplicate determinations.

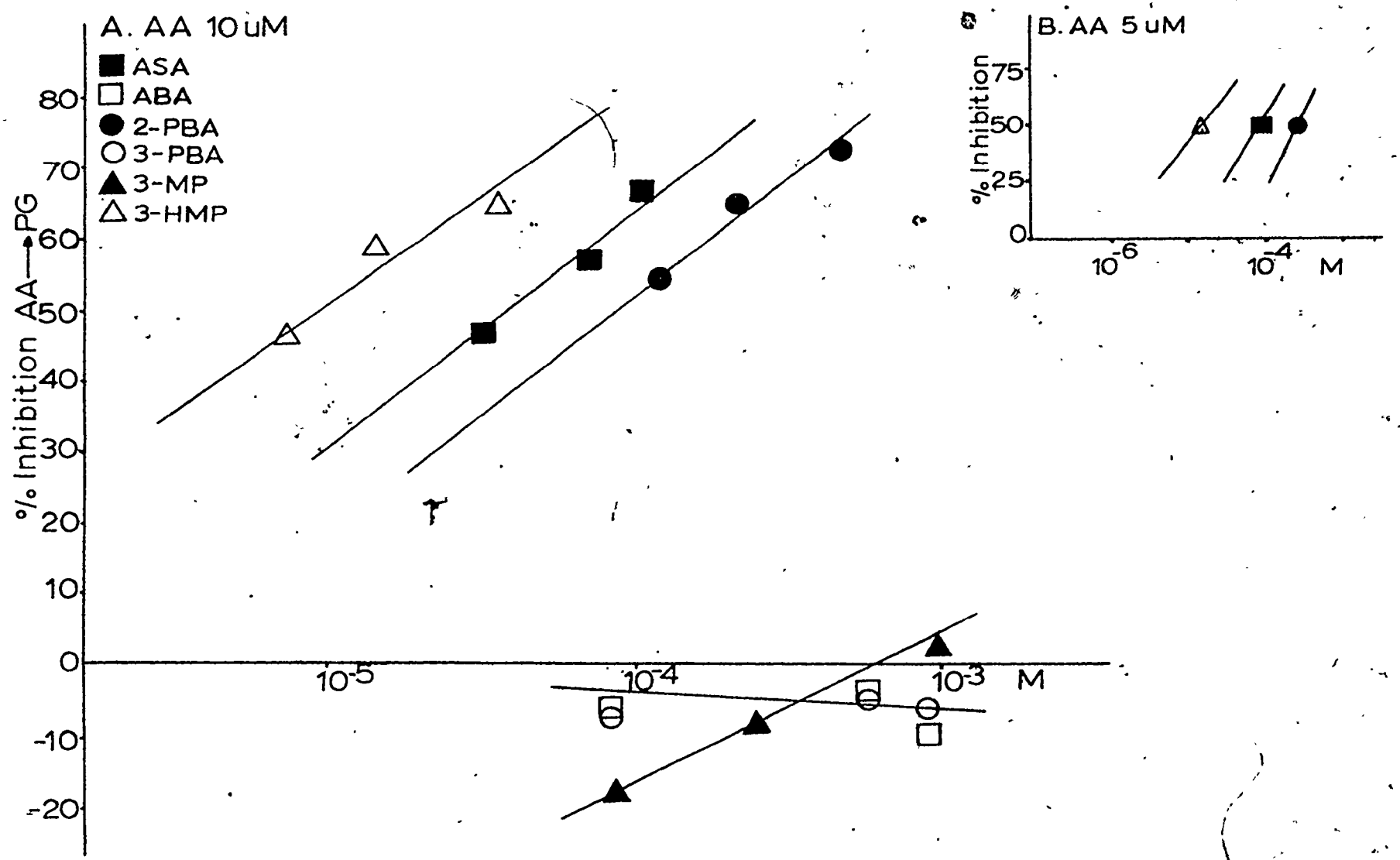
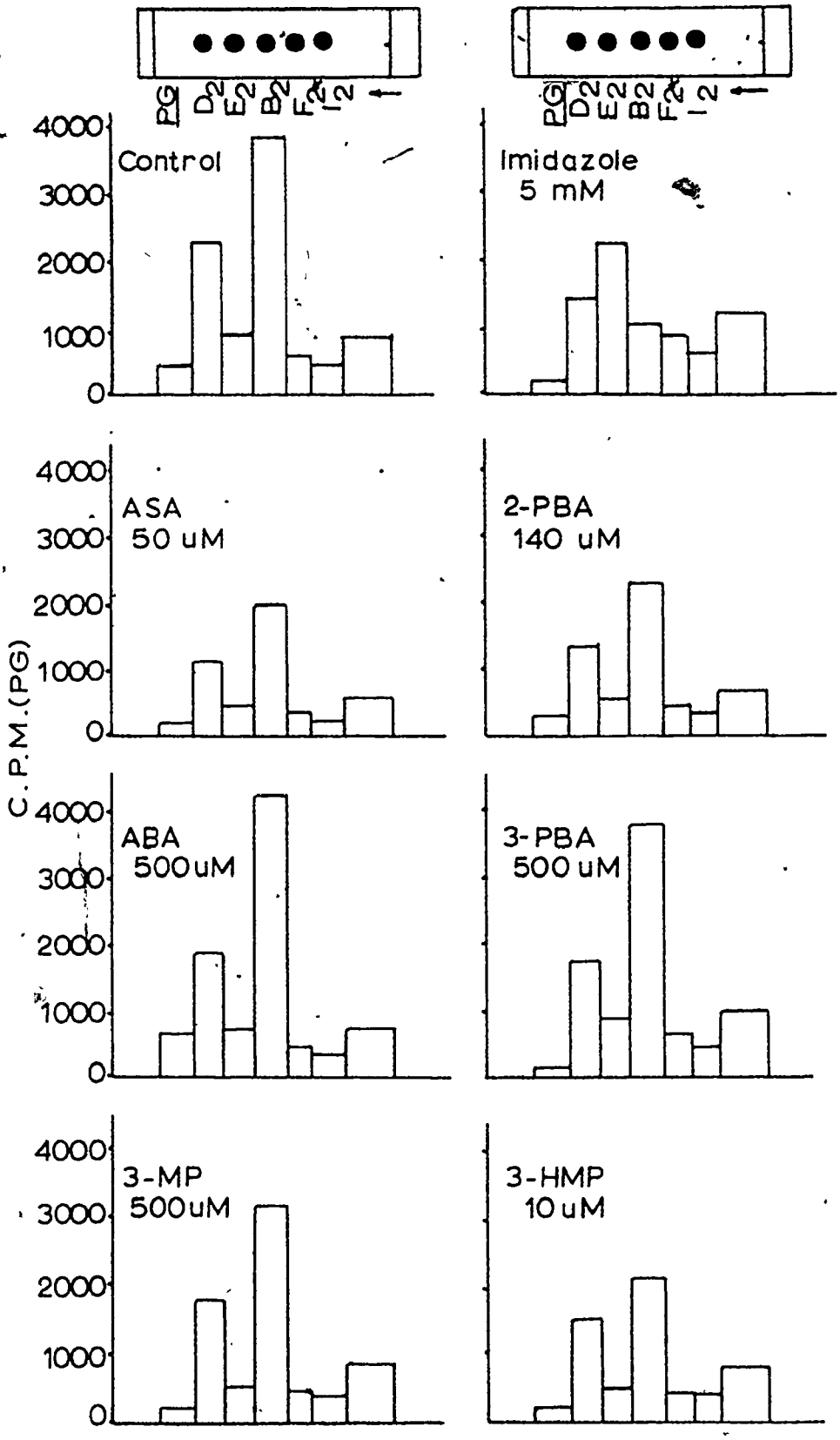

A. AA 10 uM
ASA
ABA
2-PBA
3-PBA
3-MP
3-HMP
% Inhibition AA → PG
80
70
60
50
40
30
20
10
0
-10
-20
10-5
10-4
10-3
M
B. AA 5 uM
% Inhibition
75
50
25
0
10-6
10-4
M

Fig. 10 The effects of benzoic acid analogs on the profile of PGs and TXs produced by freeze thawed platelet lysates from ^{14}C-AA.

Each graph represents TLC separation of ^{14}C-AA metabolites found in the 6% MeOH/$CHCl_3$ fraction from silicic acid column chromatography of incubate extracts. Benzoic acid analogs were preincubated with platelet lysates for 7 min before the addition of ^{14}C-AA (10 uM) for 1 min. Solvent flows in the direction of the arrow. PG standards are indicated.
Imidazole was included as a typical thromboxane synthetase inhibitor.

B_2 represents TXB_2.


PG D2 E2 B2 F2 I2
Control
Imidazole
5 mM
ASA
50 uM
2-PBA
140 uM
ABA
500uM
3-PBA
500uM
3-MP
500uM
3-HMP
10uM
C.P.M.(PG)
4000
3000
2000
1000
0

agents were used at 500uM final concentration. All agents used at the estimated ID_{50} value caused about 50% reduction in total PG production and caused approximately equal inhibition of both peaks with no concomitant increase in PGE_2. Of the other agents, only 3-MP demonstrated a trend towards inhibition but again, this was not specific for either TXB_2 or PGD_2.

3-MP and ABA were tested at concentrations up to 1mM and ABA did not demonstrate any PG synthesis inhibition while the effects of 3-MP were weak.

2.3.2.4. Discussion

The effects of these agents on total PG synthesis were reported by Cerskus (1978) and Cerskus and Philp (1981) and we have confirmed their results here. The ability of 2-PBA to act as an inhibitor of the cyclo-oxygenase enzyme demonstrates a certain lack of absolute specificity regarding the substitution at the 2 position of benzoic acid however, the importance of the ester oxygen is demonstrated by the lack of effect of ABA, and the importance of the position of the substitution was demonstrated by the lack of effect of 3-PBA. These results are in agreement with the proposed receptor template for PG cyclo-oxygenase as reported by Scherrer (1974).

The fact that 3-MP does not demonstrate significant cyclo-oxygenase inhibitory activity, in light of this receptor model and in light of the lack of effect of ABA, is not surprising. The fact that 3-MP should spontaneously photo-oxidize to a compound with a potency greater than ASA is, at the least, remarkable. Space-filling models of the benzoic acid analogs and the peroxy radical precursor of PGG_2 are shown in Plate 1.

Plate 1 Space-filling models of the benzoic acid analogs and the peroxy radical precursor of PGG_2.

Benzoic acids are oriented in such a way as to be competitive blockers of the binding site of the peroxy radical precursor of PGG_2 on the PG endoperoxide synthetase enzyme.
The central molecule is the peroxy radical precursor of PGG_2. The benzoic acid analogs are clockwise from top left: 2-PBA, ASA and 3HMP, which were all inhibitors of platelet PG synthesis, and 3-MP, ABA and 3-PBA, which had little inhibitory effect.
Analogs with PG synthesis inhibitory activity contain both oxygen atoms which coincide with acid and peroxy groups on the peroxy radical model and, chemically labile groups in this area.
This template model for NSAID was proposed by Appleton and Brown (1979).

Analogs with PG synthesis inhibitory activity, when placed in the orientation shown, contained oxygen atoms which coincided with both the acid and peroxy groups on the peroxy radical model and also, chemically labile groups in this area. This relation of benzoic acid analogs to PGs was based on the proposal by Appleton and Brown (1979).

The effect of 3HMP may relate to the hydrogen peroxide induced destruction of the enzyme as reported by Nugteren and Hazelhof (1973) and Van der Ouderaa et al (1977). 3HMP appears slightly more active than hydroperoxides reported by Nugteren and Hazelhof. Aharony et al (1981) reported the inhibition of TXB_2 and MDA production with 12-HPETE in concentration similar to those reported here. Siegel et al (1979a) ranked 12-HPETE, 11-HPETE and 9-HPETE in decreasing order of potency as inhibitors of platelet cyclo-oxygenase.

The effect of monocyclic peroxides on platelet aggregation as reported by Porter et al (1976) (see platelet aggregation discussion) are in line with these effects of 3HMP on the cyclo-oxygenase enzyme. Like 3HMP, both phases of aggregation were inhibited instead of just the second phase as typified by ASA. These agents also blocked endoperoxide induced aggregation. The mechanism of inhibition requires further investigation keeping in mind the suggestion of Charo et al (1977) that the effects of exogenously added endoperoxides may not totally represent the effects of internally generated ones.

The freeze thawed washed platelet lysate system has several features of interest in the study of platelet PG synthesis. Needleman et al (1979) claimed that in intact platelets the endoperoxides are tightly bound to the thromboxane synthetase system and that endoperoxides are released only in the presence of a thromboxane synthetase inhibitor. If

we hypothesize that in these disrupted platelets some endoperoxides are not as tightly bound and are released, the endoperoxides should decompose and leave PGE_2 as the major product with some PGD_2 (see Needleman et al 1979) because no PG endoperoxide 11-keto isomerase activity has been reported, no glutathione is added for glutathione-S-transferase activity and no serum albumin is present, all of which lead to PGD_2 formation. This is especially true in the presence of a thromboxane synthetase inhibitor. In this model, imidazole did lead to an increase in PGE_2 but also led to a decrease in PGD_2 suggesting some relation between the formation of TXB_2 and PGD_2 at least in the lysed platelet preparation (see Ali et al 1977). The importance of these results are difficult to assess as Baumgartner and Muggli (1976) reported that platelets remained intact after adhesion however, this may not be the case upon platelet accumulation in thrombus formation.

In light of these results, it would be wrong to postulate that as both PGD_2 and TXB_2 peaks are inhibited to the same degree by the inhibitory agents, that none of the agents was specific for the thromboxane synthetase enzyme. The absence of the concomitant increase in PGE_2 with the inhibition of the TXB_2 peak that is seen with imidazole, would suggest that inhibition occurs at a step involved in the production of the endoperoxides. Based on these results, 3-MP does not exert its effect on platelet aggregation through specific thromboxane synthetase inhibition.

Reports of increased platelet aggregation with ABA and 3-PBA and release of ATP with 3-PBA (see platelet aggregation) and increased production of a proaggregatory substance from platelet microsomes with 3-PBA suggest that these agents may cause increased platelet PG and

thromboxane production. Based on these results, if increased PG production does occur, it is not likely through increased activity at the cyclo-oxygenase step. This may be explored further by using a lower concentration range of 3-PBA in this preparation.

2.3.3 Effects of Benzoic Acid Analogs on Platelet Cyclic Nucleotide Phosphodiesterase Activity

2.3.3.1 Introduction

The role of cyclic nucleotides in platelet function has been discussed (see 2.1 and 2.3.2) and reviewed recently by Steer and Salzman (1980). While elevations in cAMP are generally associated with inhibition of platelet function, the role of cGMP is less clearly defined. Recently this cyclic nucleotide has also been shown to be associated with platelet inhibition (Davidson and Haslam 1981, Mellion et al 1981).

Cyclic nucleotides are metabolized primarily by the cyclic nucleotide phosphodiesterase (PDE) enzyme (EC 3.1.4.17), an enzyme found in almost all mammalian cells. The enzyme exists in several molecular forms and these forms are unequally distributed in different tissues, making this enzyme a likely site for the pharmacological manipulation of cyclic nucleotide levels (Weiss and Hait 1977). Blood platelet PDE was reported to exist in multiple forms by Amer and Mayol (1973), Pichard et al (1973) and Hidaka et al (1974). More recently Hidaka and Asano (1976a) separated three forms of the enzyme and showed that each form had various affinities for each cyclic nucleotide which in turn, were hydrolyzed at the same site. Similar work by Pichard and Cheung (1976) demonstrated the possibility of interconvertability between various enzyme forms and Hidaka and Asano (1976b) provided further evidence for this idea by showing that calcium influenced the appearance of a smaller

molecular weight PDE form at the expense of a higher molecular weight form. Ryo et al (1977) also reported platelet calcium dependent PDE activity.

The cellular regulation picture today is one of a complex interrelationship between second messenger systems involving calcium, cAMP, PGs and possibly cGMP. To some extent, this has been unified by the discovery of calmodulin. Calmodulin is a calcium binding protein which, unlike other calcium binding proteins, can confer reversible activation upon a number of enzymes including phospholipases, adenylate cyclase, some PDEs and others (see reviews by Cheung (1980), Klee et al (1980), Browstrom and Wolff (1981)). It is inhibited by phenothiazine compounds (Browstrom and Wolff 1981). Calmodulin was first reported in platelets (Smoake et al 1974) as an activator of platelet PDE. In the presence of cAMP there is increased affinity of calmodulin for the PDE (Cheung et al 1981) hence increased activity, therefore it appears the cAMP can lead to conditions favourable to its own hydrolysis. The mechanism of calcium activation of calmodulin and PDE has been reported by Huang et al (1981). In platelets heat labile and heat stable factors exist which can inhibit the effects of calmodulin on PDE (Wong and Cheung 1979). Phospholipids have been shown to activate PDE and simultaneously prevent calmodulin activation, switching off control by the calmodulin regulatory mechanism (Klee et al 1980). Calcineurin, a calcium binding protein almost exclusively associated with calcium accumulation by the endoplasmic reticulum (Browstrom and Wolff 1981), has been shown to inhibit calmodulin stimulated PDE by competing with the enzyme for calmodulin (Cheung et al 1981). This protein has not been reported in platelets as yet. Endogenous inhibitors of PDE activity have

also been reported (Pinkett et al 1979, Niles and Lowey 1981).

The pharmacological inhibition of PDE has been reviewed by Amer and Kreighbaum (1975) and Weiss and Hait (1977). The fact that the precise subcellular locations of the various PDE forms and activators are not known and the anomalous kinetics characteristic of crude enzyme assays, make inhibitor studies on these enzymes difficult to interpret. A variety of studies have tested the effects of NSAID on PDE activity. In 1970, Ball et al demonstrated that PGE_1 and ASA caused synergistic inhibition of collagen aggregation but not synergistic effects on platelet cAMP levels. Amer and Marquis (1972) reported that doses of ASA as low as 10^{-5}M caused a shift in PDE forms from a high affinity form (low Km) to a low affinity form (high Km). Schonhofer et al (1973) reported that salicylates inhibit fat cell PDE over a concentration range of 6.0×10^{-3} to 6.0×10^{-2}M. Stefanovich (1974) reported similar results with ASA and salicylate but found indomethacin to be more potent (ID_{50} 6.9×10^{-5}M). Newcombe et al (1974) tested a series of NSAID on PDE from chicken cartilage and compared these effects to the potency of the NSAID as PG synthesis inhibitors. ASA was a weak inhibitor at 10^{-3}M. Aryl acetic acids, mefenamic acid, quinoloine compounds and pyrazolone compounds all demonstrated significant inhibition. Also, these agents were usually the most potent PG synthesis inhibitors, however, the stereospecific requirements for anti-inflammatory activity and PG synthesis inhibition did not hold for PDE inhibition. More recently, Rafanell et al (1979) compared ASA and triflusal as inhibitors of platelet PDE and found ASA to inhibit the enzyme at concentrations greater than 10^{-3}M while triflusal was a more potent inhibitor.

The role of the PDE inhibitor in control of platelet function has

been accented by the theoretical interaction of these agents with the adenylate cyclase stimulating PGs especially PGI_2. Reports by Moncada and Korbut (1978) and Jorgenson et al (1979) have lent experimental evidence to this theory. PDE inhibitors affect platelets in much the same way as PGI_2. Tang et al (1980) reported that a series of PDE inhibitors blocked aggregation and shape change induced by ADP and thrombin. Papaverine and xanthines were reported to cause disaggregation of platelets independent of PGI_2 (Radomski et al 1981) and ASA actually potentiated this activity. Zahavi and Kakkar (1981) suggested that cAMP exists in separate pools and that undetectable elevations in cAMP could mediate the inhibition of platelet function while Lam et al (1981) proposed that certain PDE inhibitors worked through alternative mechanisms. Along these lines, Dunlop et al (1981) reported that caffeine and theophylline, at high concentrations, caused calmodulin dependent effects on cerebellar PDE due to calcium mobilization.

Nemecek et al (1979) and Nemecek (1981) made the observation that, in the presence of PGE_1, there was reduced cAMP PDE activity in fibroblast homogenates. These data suggested that prolonged accumulation of cAMP induced by PGE_1 was mediated partially through PGE_1 inhibition of platelet PDE. This finding is of interest in light of the report by Gorman et al (1977a) that PGI_2 maintained elevated levels of cAMP in platelets longer than PGE_1. An endogenous PDE inhibitor would account for such a prolonged effect.

Certain common factors exist between the PG-thromboxane system and the cyclic nucleotide PDE system. ASA causes the inhibition of PG synthesis and a shift of forms of PDE. Indomethacin inhibits PG synthesis and PDE as well as other enzymes. Adrenaline induces the shift

of PDE enzymes to the high affinity form (Amer and Marquis 1972) and enhances cyclo-oxygenase activity (first shown by Takeguchi et al 1971). Both imidazole and nicotinic acid are activators of PDE activity and are inhibitors of thromboxane synthetase (Vincent and Zijlstra 1978). Calmodulin provides a further link between the two systems.

McElroy and Philp (1975) reported that a series of dipyridamole-like drugs inhibited ADP-induced platelet aggregation in a rank order of potency reflected in the relative potency of each agent as an inhibitor of cAMP PDE compared to cGMP PDE. This work provided further evidence that cAMP and cGMP mediated opposing cellular events in the platelet. Using a series of methylxanthines, which proved to be less potent PDE inhibitors, especially on cGMP PDE, Killackey (1978) showed a correlation between the inhibition of ADP-induced platelet aggregation and the inhibition of high affinity cAMP PDE activity.

In this study, the effects of the ASA-like drugs on platelet PDE activity were examined based on the following facts:

1. some NSAID inhibit PDE activity (see above)
2. there is a similarity in the effect of 3-MP and 3HMP on platelet aggregation compared to known PDE inhibitors (Philp et al 1973, McElroy and Philp 1975) and
3. there is a similarity in structure between 3-MP and 3HMP and certain anticoagulants such as dicoumarol, which inhibits platelet PDE (Amer and Kreighbaum 1975).

Brief studies were also undertaken to test for the production of an endogenous PDE inhibitory activity induced in PGI_2-inhibited platelets and to assess the involvement of calmodulin in PDE activity by using the calmodulin inhibitor trifluoperazine. This agent has been shown to

inhibit calmodulin activity (Levin and Weiss 1976) and inhibit platelet function (Kindness et al 1980, White and Raynor 1980).

2.3.3.2 **Methods**

Cyclic nucleotide PDE activity from human blood platelets was measured in PRP by a method recently published by Thompson et al (1979). In this assay, tritiated cyclic nucleotide is hydrolyzed to the noncyclic monophosphate by the PDE enzyme(s) in platelets (Fig. 11A). The product is separated from substrate by conversion to the uncharged nucleoside using the 5'-nucleotidase found in King Cobra snake venom (Ophiaphagus hannah). The incubation mixture is applied to an anion exchange column where the uncharged product is separated from the charged nucleotides. The product is collected and counted by liquid scintillation counting. ^{3}H-cAMP 26 Ci/mmol and ^{3}H-cGMP 19 Ci/mmol were obtained from Amersham Searle (Oakville, Ontario). Isotopes which were not used within 5 weeks were purified on cellulose thin layer plates (Eastman Chromogram Cellulose) by double development with 2-propanol/ NH_4OH/H_2O (7:1:2 v:v:v). Cyclic nucleotides were eluted and stored at -20° C in 50% ethanol in a multidose vial. Snake venom (Sigma Chemical Co., St. Louis) was made up as a 1 mg/ml solution and stored at 4° C for less than 8 days. The anion exchange resin (Biorad AG-1X2, 200-400 mesh, Bio-Rad Laboratories) was washed with 0.5N NaOH, double distilled water, 0.5N HCl and then double distilled water until the test for chloride ion was negative and the pH was 5. This was stored at 4° C in double distilled water in a ratio of 2 volumes water to 1 volume resin. All pipettings were critical and were made with Oxford Samplers except for the radioactive nucleotides which were measured with a 10ul Hamilton

Syringe (Hamilton Co., Reno).

Eppendorf Microtest tubes (Fisher Scientific Co., Toronto) were used for incubations and into these were placed 50ul 20% DMSO (in millipore-filtered saline) or drug made up in this solution, 50ul ^{3}H-cyclic nucleotide and one of a variety of concentrations of nonlabelled cyclic nucleotide to cover the substrate concentrations desired. The amount of labelled substrate was such that each sample contained 1.5×10^{5} CPM. At 30 second intervals, 250ul freeze thawed PRP (PRP was stored at -20° C for 1-7 days) in buffer or saline buffer were added to each Eppendorf to initiate the reaction. These buffer solutions were made up with PRP or saline in a 1:4 ratio with Tris-HCl buffer (0.04M pH 8.0 containing 0.02M $MgCl_2$ and 4.67mM 2-mercaptoethanol) and were made up fresh each day. The reaction progressed under continuous agitation, in a Thermolyne 5900 Dry Bath at 37° C for various times. The reaction was terminated by placing the test tubes in a boiling water bath for 30 seconds and then on ice.

The 5'-nucleotidase step involved the incubation of each sample with 100ul snake venom solution for 10 minutes at 30° C in a Themolyne DB12215E Dry Bath. The samples were then returned to ice until all incubations were complete. Methanol (1 ml) was added and mixed with the sample by a brief vortex and the samples were spun for 2 minutes in an Eppendorf 3200 Centrifuge (Brinkman Instruments, Toronto). The samples were then returned to ice.

The column method of anion exchange separation of nucleotides was used (Thompson et al 1974). Glass columns 0.6cm x 14.6cm (Pasteur pipettes, Fisher Scientific Co., Toronto) were plugged with glass wool plugs and anion exchange resin was added to a height of 1.5cm in the

column. The resin was not allowed to dry. The entire supernatant from each sample was placed on the column and the reaction mixture was eluted with 1 ml methanol. The column was allowed to drain to dryness into plastic scintillation vials and 10 ml of ScintiVerse Universal Liquid Scintillation Cocktail (Fisher Scientific Co., Toronto) was added to each vial and the solutions mixed. All samples were counted on a Searle Diagnostic Model 6892 liquid scintillation counter and CPM were converted to disintegrations per minute (DPM) by the external standards ratio method (ESR).

Substrate concentrations were chosen to reflect the enzyme activities found in platelets. Time course studies were performed on all substrate concentrations to ensure linearity and optimal substrate conversion within the chosen incubation time period. Because of the multiple enzyme systems present, ID_{50} values were determined as an indicator of drug inhibitory potency. The application of Michaelis-Menton kinetics to these conditions is questionable.

The inhibitory effect of trifluoperazine (0.5 mg/ml or 1.5×10^{-4} M) was used as an indication of calmodulin regulation of the enzyme. Trifluoperazine was incubated with PDE under conditions similar to the ASA-like drugs.

2.3.3.3 **Results**

Freeze thawed PRP yields at least 3 distinct PDE activities (Fig. 11). Previous work in our laboratory demonstrated 2 cAMP PDE activities (Fig. 11A), a high affinity (Km= 0.50uM) and a low affinity activity (Km >100uM) and one cGMP activity (Km= 1.5uM). Anomalous kinetic curves for both cAMP and cGMP PDEs have been reported and because we wished to compare inhibition potencies of these benzoic acid analogs, we chose to compare ID_{50} values. Data is plotted as % inhibition versus inhibitor concentration in mg/ml to facilitate the extrapolation of any inhibitory effect to the <u>in vivo</u> situation (Fig. 12). 1-methyl-3-isobutylxanthine (MIX)(0.055 mg/ml or $2.5x10^{-4}$M final concentration) was included in all studies to serve as a control test for enzyme inhibition. Assays were set up so that 10-30% of the substrate was metabolized within the time interval chosen. The high affinity cAMP PDE was studied using 0.2uM substrate concentration and a 5 minute incubation period. 3-MP inhibited the high affinity cAMP PDE activity with an ID_{50} value of $1.14x10^{-3}$M (Fig. 12A). Inhibition was evident at concentrations as low as $1x10^{-4}$M. No other agent caused significant inhibition over the concentrations tested and higher concentrations were found to overcome the buffering capacity of this system.

These agents were tested for effects on the low affinity cAMP PDE enzyme using a 250uM substrate concentration and a 45 minute incubation period (Fig. 12B). Under these conditions the ID_{50} for 3-MP was $3.5x10^{-3}$M. 3HMP caused inhibition at the highest concentration tested here (ID_{25} $8.3x10^{-4}$M). No other agents caused inhibition of this enzyme. A brief study was also carried out to examine these agents as inhibitors

Fig. 11 The kinetics of cAMP and cGMP metabolism by the cyclic nucleotide phosphodiesterase activity (PDE) of freeze thawed platelet lysates.

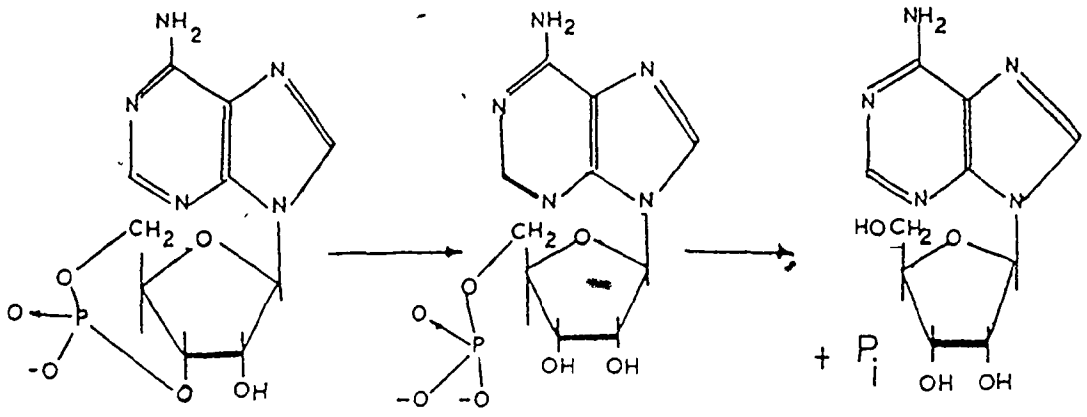
The upper figure represents the reaction scheme for the assay of cyclic nucleotide metabolism by PDE.

A. Eadie Plot (100 v/s versus v) of cAMP PDE activity in freeze thawed platelet lysates.
Km I = 228.4 uM Km II = 0.51 uM

B. Eadie Plot of cGMP PDE activity in freeze thawed platelet lysates.
Km = 1.49 uM

s= substrate concentration in uM
v= nanomoles of substrate hydrolyzed per 50 ul PRP per 5 min.

from Killackey (1978).



Cyclic [^{3}H] 3',5'-AMP $\xrightarrow[\text{step 1}]{\text{PDE}}$ [^{3}H] 5'-AMP $\xrightarrow[\text{step 2}]{\text{snake venom}}$ [^{3}H] adenosine

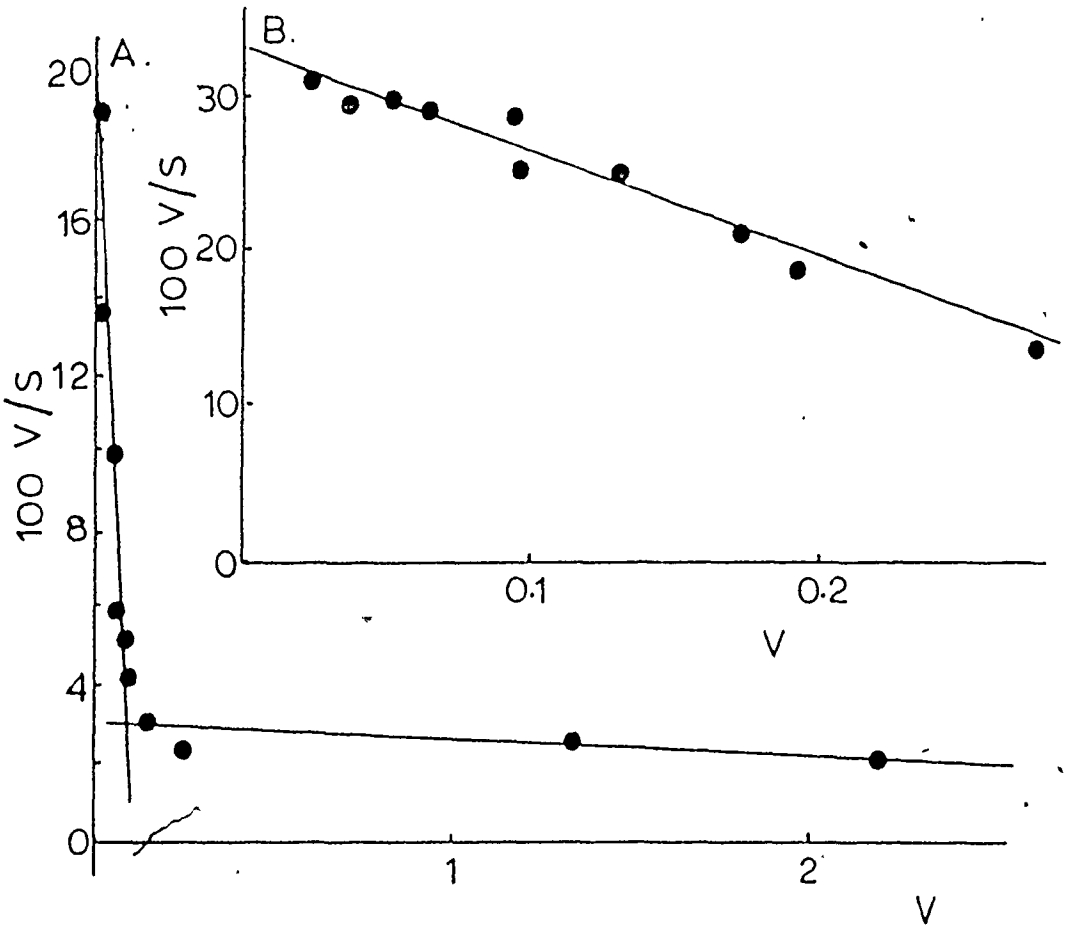


Fig. 12 Dose-response effects of the benzoic acid analogs on cyclic nucleotide metabolism by freeze thawed platelet lysates.

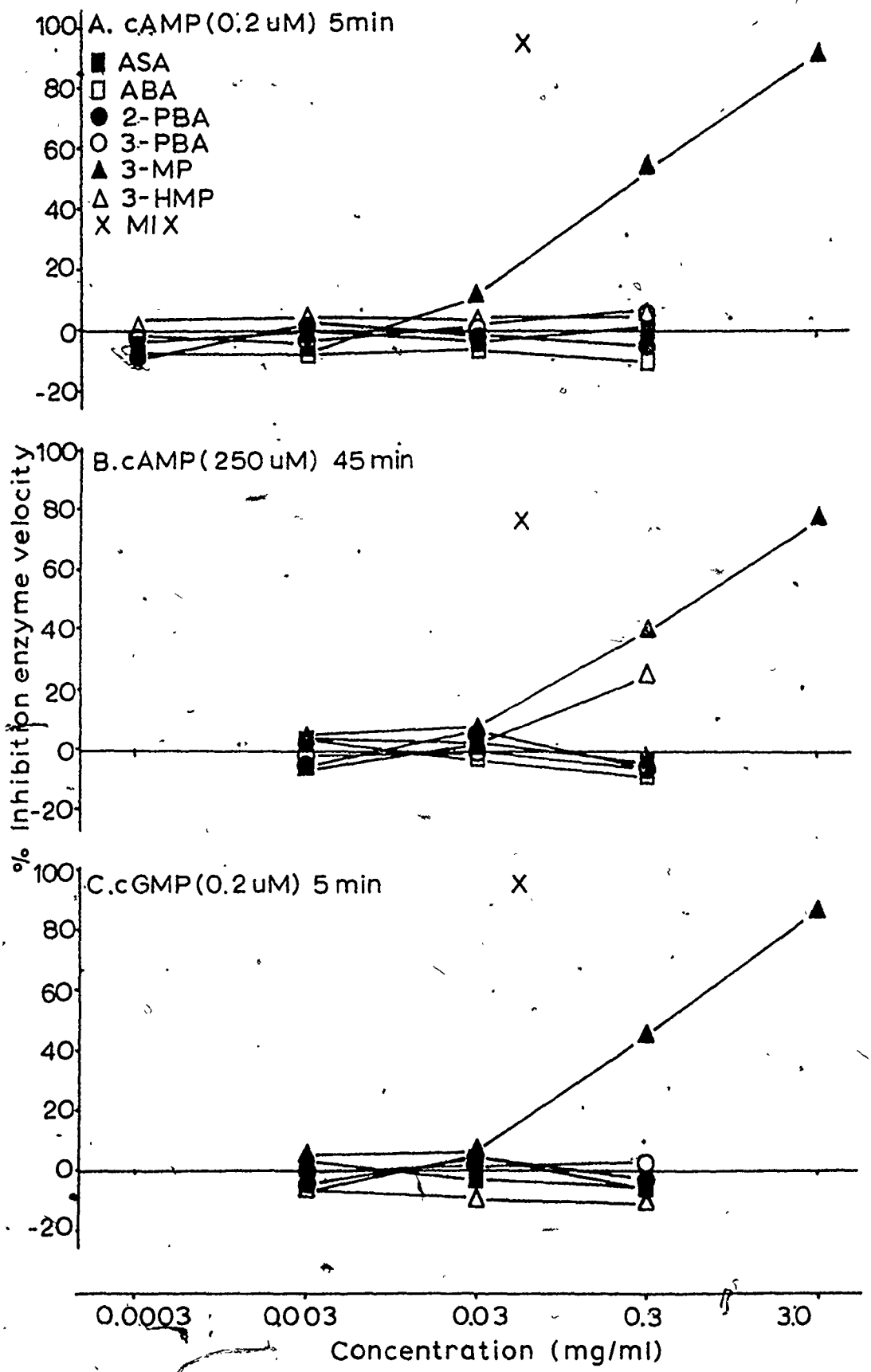
% Inhibition of enzyme velocity was calculated by comparison with controls in which buffer was added instead of benzoic acids. Each point represents the average of triplicate determinations.
1-methyl-3-isobutylxanthine was included in each assay as a control.

A. % Inhibition of cAMP high affinity PDE. cAMP concentration = 0.2 uM. Reaction time = 5 min
ID_{50} 3-MP = 1.14×10^{-3} M.

B. % Inhibition of cAMP low affinity PDE. cAMP concentration = 250 uM. Reaction time = 45 min
ID_{50} 3-MP = 3.5×10^{-3} M.

C. % Inhibition of cGMP PDE activity. cGMP concentration = 0.2 uM. Reaction time = 5 min
ID_{50} 3-MP = 2.5×10^{-3} M.

0.3mg/ml = 2mM 3-MP.


A. cAMP (0.2 uM) 5min
ASA
ABA
2-PBA
3-PBA
3-MP
3-HMP
MIX
B. cAMP (250 uM) 45 min
C. cGMP (0.2 uM) 5 min
% Inhibition enzyme velocity
100
80
60
40
20
0
-20
0.0003
0.003
0.03
0.3
3.0
Concentration (mg/ml)

of the cGMP PDE activity. Conditions were similar to those for the high affinity cAMP PDE enzyme. Again 3-MP was the only agent to show significant inhibition (ID_{50} $2.5x10^{-3}M$) (Fig. 12C) and did so at an ID_{50} value similar to that found for other enzymes. 3HMP had no significant effect here, nor did the other agents. In all these studies, drugs were dissolved in 20% DMSO giving a final incubation conentration of 2.5% DMSO. Controls were run in the presence of this concentration of DMSO. Because of the unknown stability of 3HMP in DMSO, 3HMP was dissolved in saline and the results were compared to saline controls. No drugs inhibited the 5'nucleotidase at the highest concentrations used here.

Brief studies were carried out to determine the involvement of calmodulin in the activity of the various PDE enzymes in this freeze thawed platelet preparation (Fig. 13). When trifluoperazine ($1.5x10^{-4}M$), a calmodulin inhibitor, was incubated with freeze thawed platelets under the conditions described above, there was 36% inhibition of the high affinity cAMP PDE activity, 16% inhibition of low affinity cAMP PDE activity and no inhibition of cGMP PDE activity. Although these results are preliminary, they suggest that cAMP PDE activity is under calmodulin regulation whereas the cGMP PDE, at least under the conditions of these assays, is not calmodulin dependent.

Another brief study was designed to assay the effects of prostacyclin (PGI_2) on PDE activity of our systems. In this study, 7.5uM PGI_2 was added to PRP under the following conditions: (1) 2 minutes before freeze thawing (to intact platelets) (2) after freeze thawing (to lysed platelets) (3) only in the buffer solution or (4) not at all. 7.5uM PGI_2 was also included in the buffer of (1) and (2) to ensure that it was present at all times to avoid any possible washout effect

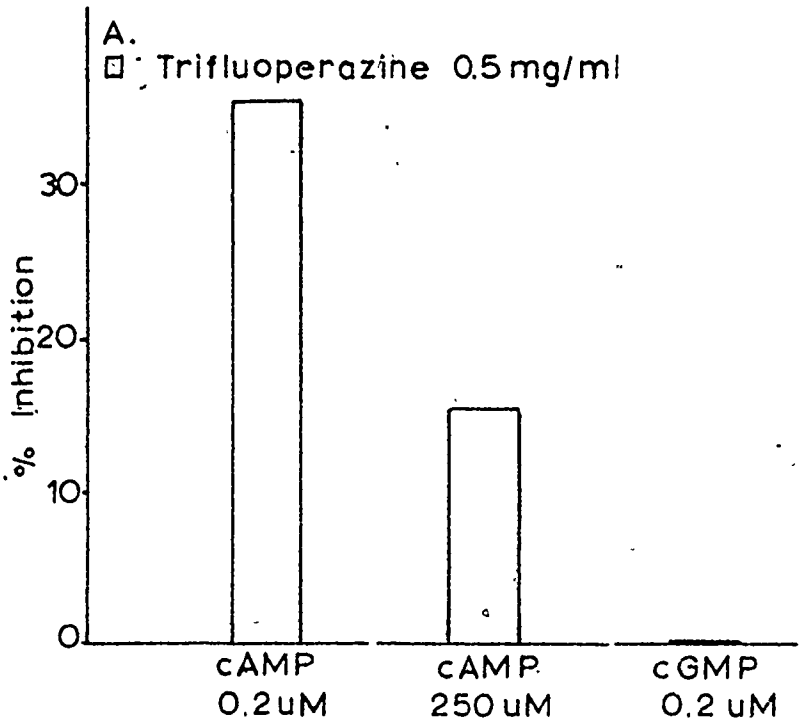
Fig. 13 Additional studies of platelet PDE activity.

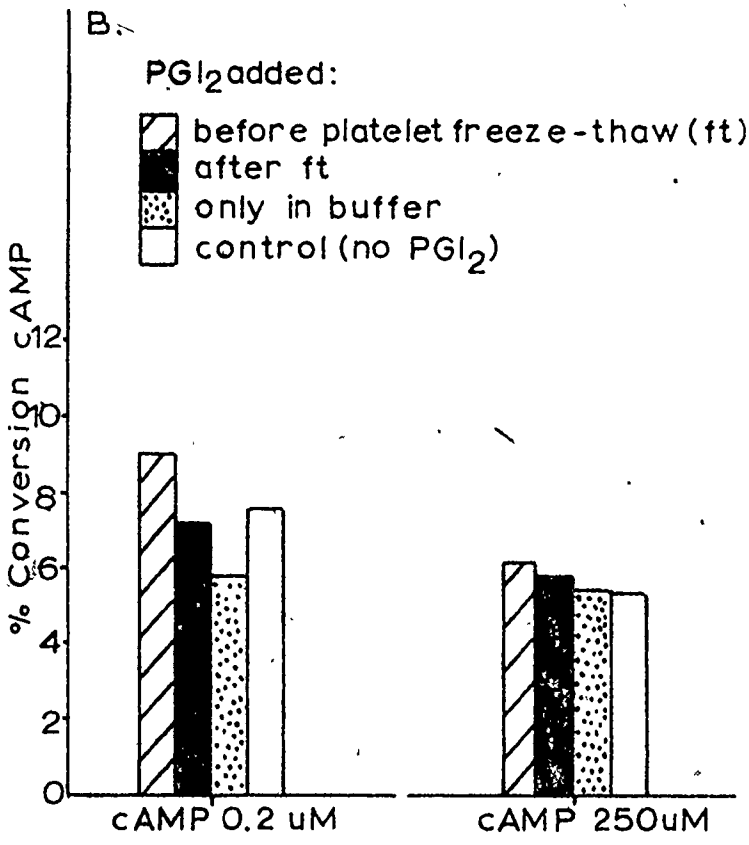
A. The effects of the calmodulin inhibitor trifluoperazine (0.5 mg/ml) on platelet PDE activity.

Trifluoperazine was incubated with platelet lysates with various concentrations of cAMP, to reflect the different cAMP and cGMP PDE activities. Conditions include 0.2 uM cAMP with a 5 min reaction time, 250 uM cAMP with a 45 min reaction time and 0.2 uM cGMP with a 5 min reaction time. Bars represent the % inhibition of each activity as compared to controls in which no trifluoperazine was added. Each bar is the average of triplicate determinations.

B. The effects of PGI_2Na^+ on platelet PDE activity.

PGI_2Na^+ was added at various times in the preparation of freeze thawed platelet lysates to examine possible effects of PGI_2 on cAMP high and low affinity PDE activity. PGI_2Na^+ (7.5 uM) was added to whole platelets before freeze thawing, immediately after freeze thawing, only in the assay buffer or not at all. Except in the last condition, PGI_2Na^+ (7.5 uM) was included in all assay buffers. Graph represents the % conversion of cAMP to 5'-AMP by PDE activity under the conditions of 1. cAMP 0.2 uM substrate concentration with a 5 min reaction time or 2. 250 uM cAMP with a 45 min reaction time.


A.
Trifluoperazine 0.5 mg/ml
% Inhibition
30
20
10
0
cAMP 0.2uM
cAMP 250 uM
cGMP 0.2 uM


B.
PGI2 added:
before platelet freeze-thaw (ft)
after ft
only in buffer
control (no PGI2)
% Conversion cAMP
12
10
8
6
4
2
0
cAMP 0.2 uM
cAMP 250uM

(Nemecek et al 1979). Other than these modifications, all PRP was treated the usual way and assessed for PDE activity under high affinity (cAMP= 0.2uM, 5 minute incubation) and low affinity (cAMP= 250uM, 45 minute incubation) conditions (Fig. 13).

There were no dramatic effects of prostacyclin on platelet PDE apparent in this experiment. There was a trend however, towards an increase in cAMP activity, under high affinty conditions when PGI_2 was added prior to the freeze-thaw procedure. There was a trend towards inhibition of high cAMP activity when prostacyclin was only included in the buffer. Under low affinity cAMP PDE conditions, PGI_2 was not stable. No effects were seen here.

2.3.3.4 **Discussion**

The anomalous kinetics displayed by cAMP PDE are a common feature of PDE activity in crude preparations. Anomalous kinetic plots may also be obtained for the cGMP PDE activity if the enzyme concentration is altered (Pichard and Cheung 1976). This prevents accurate interpretation of inhibitor kinetic data as this type of kinetics could represent two enzyme forms or negative co-operative effects (Weiss and Hait 1977). By assaying drug effects in this crude preparation under different substrate and incubation conditions, we are not attempting to extrapolate these effects to separate enzymes, but to separate enzyme activities.

This method, a recent modification of the widely used PDE assay method of Thompson et al (1974), overcomes the problems of nonspecific nucleotide binding to the anion exchange resin (Thompson et al 1979). In this assay, recovery of adenosine is greater than 95% with less than 1%

cAMP in the blank. Recoveries of guanosine were somewhat less than adenosine (Thompson et al 1979). DMSO has been used as a solvent in these studies and the final concentration of 2.5%, which is less than that of Kramer et al (1977), did not significantly change enzyme activity. In each study MIX, 2.5×10^{-4}M, was included to ensure that the enzyme activity was subject to inhibition under the usual conditions. MIX was a potent inhibitor of all enzyme activities.

The ASA-like agents in this study, that exist in a "chain" conformation (ASA, 2-PBA, 3-PBA, ABA partially), had no effects on PDE activity at the concentrations tested. This is not surprising in view of the cited literature on the effects of salicylates on PDE (Introduction). In contrast, 3-MP, which is a "ring" or bicyclic compound, inhibited all PDE activity under all assay conditions. This agent has structural similarities to the purine nucleus suggesting some competitive inhibitory effect but the structure also resembles certain oral anticoagulants which are known to have PDE inhibitory activity (Amer and Kreighbaum 1975). There may be further chemical pathways in common in the coagulation of blood and the function of the blood platelet, as suggested by the effects of the coumarin anticoagulants on vitamin K epoxide reduction (O'Reilly 1980), platelet PDE and possibly PG endoperoxide metabolism.

The inhibition of the high affinity cAMP PDE activity by 3HMP may result from its similarity in structure to 3-MP and the less rigid structural requirement for inhibition of this activity. Alternatively, this compound may inactivate the enzyme through some mechanism involving its highly reactive peroxy group. No test was made for enzyme destruction but, if such a phenomenon did occur, it would most likely

occur under these long incubation conditions.

The inhibition of platelet PDE enzymes over the concentration range found to inhibit platelet aggregation, provides a ready explanation for the inhibition of both phases of ADP aggregation by 3-MP, unlike PG synthesis inhibitors. 3-MP caused good PDE inhibition and weak PG synthesis inhibition while 3HMP had the opposite effect. The effects of 3-MP resemble the effects of triflusal (Rafanell et al 1979) which is a structural analog of ASA, is a weaker cyclo-oxygenase inhibitor but a more potent PDE inhibitor and demonstrates better in vivo antithrombotic activity in a rat thrombosis model. The inhibition of cGMP PDE and cAMP PDE over the same concentration range would provide evidence against these nucleotides mediating opposing cellular events.

The inhibition of both phases of aggregation by 3HMP cannot be explained in terms of PDE inhibition or PG synthesis inhibition and this phenomenon requires further investigation.

Results with trifluoperazine are preliminary but suggest that both cAMP PDE activities are under some degree of calmodulin regulation. This would be in line with the role of cAMP in the control of intracellular calcium concentration as suggested by White and Gerrard (1978)(see 2.3.2.1). The lack of cGMP inhibition by trifluoperazine does not rule out the control of some other cation by this cyclic nucleotide.

Results of the preliminary experiments testing PDE activity in PGI_2 stimulated platelets appear analogous to reports of Nemecek et al (1979) using PGE_1 and fibroblasts. They found that removal of the PGE_1 before PDE assay resulted in an apparent increase in PDE activity in sharp contrast to the inhibition of activity seen when the agonist was included in all washing and assay media. Although attempts were made to

include PGI_2 in all parts of the assay, the instability of this PG compared to PGE_1 may have counteracted these attempts. This study was prompted, in part, by a lack of synergistic activity between a series of known PDE inhibitors and PGI_2Na^+ (results not shown). This phenomenon merits further investigation for the understanding of the pharmacological effects of PGI_2 in relation to cAMP.

This work demonstrates that although ASA and other ASA-like chemicals in the chain conformation do not inhibit PDE activity, ring conformation compounds, especially 3-MP inhibit this enzyme. This is proposed as the mechanism of inhibition of platelet activity by 3-MP. Because the effects of 3HMP on platelet aggregation cannot be totally explained in terms of PDE inhibition or PG synthesis inhibition, further investigation here may uncover new mechanisms of mediating inhibition of platelet function.

2.4 In Vitro Effects of Benzoic Acid Analogs on Vessel Wall Prostaglandin and Prostacyclin Production

2.4.1 Effects of Benzoic Acid Analogs on Prostacyclin-like Activity by the Blood Vessel

2.4.1.1 Introduction

Prostacyclin (PGI_2) is the most potent, naturally occurring inhibitor of platelet aggregation. It is chemically unstable due to a 5,6 enol ether linkage which degrades to form 6-keto $PGF_{1\alpha}$, a stable but relatively inert compound (Higgs et al 1980). The $t_{1/2}$ of PGI_2 is about 10 minutes at 22° C and neutral pH. Boiling destroys the activity within 15 seconds and the $t_{1/2}$ increases to 100 hours at pH 10.5 at 25° C. PGI_2 causes many pharmacological effects (see Vane and Bergstrom 1980, Moncada and Vane 1981).

PGI_2 inhibits platelet aggregation at a much lower concentration than necessary to prevent adhesion (Higgs et al 1980) and it can also disaggregate platelet clumps in vitro (Ubatuba et al 1979). PGI_2 inhibits platelet aggregation induced by all stimuli including platelet activating factor (PAF) which works through different mechanisms from the other common agonists (Bussolino and Camussi 1980). PGI_2 inhibits platelet shape change, the simultaneous development of procoagulant activity (Ehrman and Jaffe 1980) and the platelet mobilization of fibrinogen binding sites (Hawiger et al 1980). PGI_2, with preincubation, inhibited platelet aggregation with an ID_{50} of 7pmol/ml (Whittle et al

1978). It was reported to be more effective on platelet aggregation by suboptimal doses of ADP and no inhibition was observed, in a sensitive assay system, at PGI_2 concentrations less than 0.4pmol/ml (Haslam and McClenaghan 1981). Haslam and McClenaghan (1981) reported that PGI_2 was rapidly cleared from the plasma and this was in agreement with Wong et al (1978) who reported the rapid termination of PGI_2 action by such routes as the blood vessel enzyme 15-hydroxy PG dehydrogenase and by 9-hydroxy PG dehydrogenase of platelets (Wong et al 1980) among others (Korff and Jarabek 1981). Although the $t_{1/2}$ for PGI_2 is relatively short, Pederson (1978), Gimeno et al (1980), Wynalda and Fitzpatrick (1980) and Borda et al (1980) reported long lasting biological effects and various proposals including albumin stabilization, platelet PDE inhibition or active metabolite formation, were put forward to explain this. Wong et al (1980) reported that PGI_2 was metabolized to 6-keto PGE_1, a compound with activities similar to PGI_2 but much more stable. Steer et al (1980) reported that antibodies to PGI_2 did not shorten the "lag time" or time between the collection of blood by venipuncture and the time of optimal platelet aggregation (usually 20-30 min). Haslam and McClenaghan (1981), reported that antibodies do not usually discriminate between 6-keto $PGF_{1\alpha}$, PGI_2 and 6-keto PGE_1, and therefore only the PDE inhibitor hypothesis for prolonged PGI_2 effect is still tenable if PGI_2 plays a role at all in the lag time effect seen in platelet function studies.

Moncada et al (1977) reported that PGI_2 synthetase activity was most highly concentrated on the intimal surface of the blood vessel and it decreased towards the adventitial surface. Proaggregatory materials were reported to be distributed in a directly opposite pattern.

Silberbauer et al (1978) reported that the subendothelium could produce some PGI_2 and Larrue et al (1980) and Jaffe (1981) reported that smooth muscle cells also produce PGI_2 but this capacity was reduced in persons with atherosclerosis (Larrue et al 1980). According to Sinzinger et al (1980) and Eldor et al (1981) the endothelium produces the major portion of PGI_2 at the luminal surface, however, other layers acquire this characteristic with time and Ingerman-Wojenski et al (1981b) reported that severe endothelial damage led to a complete loss of PGI_2 production capacity in vivo. Claeys et al (1981) have shown that chicken aorta does not produce PGI_2 and this corresponds to the lack of PGI_2 effect on chicken platelets. This important discovery suggests that other PGs such as PGE_2 may replace PGI_2 (Bult et al 1981).

Apart from the vasculature, the suggestion has been made that white blood cells also contribute to PGI_2 production in the blood (Blackwell et al 1978, Flower and Cardinal 1979).

The source of PG endoperoxide substrate for PGI_2 production is a matter of controversy (Higgs et al 1980). This stemmed partially from the reports of Gryglewski et al (1976) that the whole cell system in arterial walls converted only small amounts of exogenous AA to PGI_2 in comparison to PG endoperoxides, which are produced in abundance by platelets. Needleman et al (1978), on the other hand, reported that AA was the preferred substrate. This and other experimental evidence has led to a conflict, the resolution of which is important in the design of antithrombotic therapy. Regardless of the source, PGI_2 synthetase requires strict structural conformation of the endoperoxide as Wlodawer and Hammarstrom (1980) demonstrated that aorta did not convert PGH_1 to PGI_1. Like TXA_2 synthetase, it did convert PGH_1 to 12-

hydroxyheptadecadienoic acid (HHD) which provides a link in the mechanism of action of these enzymes. In contrast however, PGI_2 synthetase is sensitive to destruction by various peroxides while TXA_2 synthetase is resistent (Ham et al 1979). Although 2-aminomethyl-4-t-butyl-6-iodophenol prevented the destruction by various oxidants, these workers have shown that PGG_2 destruction of this enzyme is neither sensitive to this antioxidant nor time dependent indicating the unique role of AA endoperoxides.

In vivo, unstimulated arteries do not synthesize detectable amounts of PGI_2 according to Haslam and McClenaghan (1981) and Ingerman-Wojenski et al (1981b) but stimulation of PGI_2 production has been demonstrated with angiotensin II (Silberbauer et al 1979), platelet derived growth factor (PDGF) (Coughlin et al 1981) and PAF (Test and Bang 1981). Thrombin increases PGI_2 production (Whiting et al 1980) and the ASA sensitivity of this action may lead to a reevaluation of the effects of ASA on PGI_2 production.

Intriguing work by Hopkins and Gorman (1981) point to an important role of cAMP and the fine control of cAMP levels both by adenylate cyclase and PDE enzymes in endothelial cells in the control of PGI_2 synthesis and release. This work opens the door to further complex feedback interactions between released PGs, platelets and drugs in PGI_2 production. Further studies using PDE inhibitors reveal that thrombin-induced refractoriness to the release of PGI_2 must be mediated by more than PGI_2 feedback and Adams-Brotherton and Hoak (1981) suggested calcium stores are involved.

Despite the controversy over the source of substrates, Bunting et al (1976) demonstrated that fresh arterial rings, incubated in Tris for

short periods, produce PGI_2 and hence cyclo-oxygenase activity must be present and is likely subject to the usual controls (see 2.3.2). The sensitivity of the cyclo-oxygenase of different tissues to ASA has been shown to vary (Patrono et al 1976) and the suggestion has been made that the platelet cyclo-oxygenase may be more sensitive than endothelial cyclo-oxygenase. Baenziger et al (1979) reported that various blood vessel cells contained cyclo-oxygenases that were 14-44 fold less sensitive to ASA than platelets and Schror et al (1980) reported this also to be the case with inhibition by indomethacin and three fenamate NSAID. In contrast, Parks et al (1981b) reported that ASA and ibuprofen displayed similar activity on both enzymes as did Jaffe and Weksler (1979). These latter researchers reported that the effect of ASA was rapidly reversed after removal of ASA and others have shown that this depends on protein synthesis (Czervionke et al 1978, Kelton et al 1978b) whereas the platelet enzyme, as noted previously, remains inactivated for the lifetime of the platelet. Tranylcypromine was reported to be a weak but specific inhibitor of PGI_2 synthetase (Gryglewski et al 1976) however Rajtar and de Gaetano (1979) demonstrated cyclo-oxygenase inhibition with this agent. Blajchman et al (1979) reported that hydrocortisone inhibited PGI_2 synthesis at a lower dose than required to inhibit TXA_2 synthesis and nicotine has also been shown to decrease PGI_2 (Wennhalm 1980).

Various reports suggest that rat arterial tissue produces more PGI_2 than veins and Wong et al (1981) explained this phenomena as due to the greater amount of PGI_2 metabolizing enzymes in the veins. Vermylen et al (1979) suggested that Bay g 6575 and dipyridamole potentiated PGI_2 activity by inhibiting these enzymes but the report that pentoxifylline,

(like dipyridamole a PDE inhibitor), also potentiates PGI_2 release (Weithmann 1981) suggests that PDE inhibition is involved in this effect. Conflicting reports by various investigators leave the role of unsaturated fatty acid precursors unclear. Hornstra et al (1981) reported that cod liver oil, which contains a precursor to the 3-series PGs (eicosapentanoic acid), fed to rats depressed TXA_2 and PGI_2 production with no concomitant TXA_3 or PGI_3 production while Hamazaki et al (1981) demonstrated that eicosapentanoic acid treated rats produced more PGI_2 (see also Needleman 1980).

Hoult and Page (1981) reported that 5-aminosalicylic acid, like other phenolic compounds, enhanced PGI_2 formation by acting as a free radical scavenger. Prior to this work, Gryglewski et al (1976) reported that 15-hydroperoxy AA (15-HPAA) was a potent inhibitor of PGI_2 synthetase, as was spontaneously oxidized AA. They reported that 12-hydroperoxy AA (12-HPAA) was not effective at concentrations of up to 100ug/ml however, Salmon et al (1978) reported that lipid peroxides were potent inhibitors of PGI_2 synthetase. Turk et al (1980), using a platelet lipoxygenase source, demonstrated that the 12-HPAA generated was a potent inhibior of PGI_2 synthetase although the corresponding hydroxyacid was not. Gryglewski et al (1976) suggested that permeability barriers and enzymes such as glutathione peroxidase in the plasma accounted for the decreased activity of hydroperoxides on intact aorta rings in plasma compared to aorta microsomes. Weiss et al (1979) provided evidence that 15-HPAA inactivated the PGI_2 synthetase through the heme mediated generation of the hydroxyl radical ($\cdot OH$), the most powerful oxidant known. This was formed independently of the superoxide anion radical ($O_2^{\cdot -}$) or hydrogen peroxide (H_2O_2) and the enzyme could be

protected by tryptophan. The inhibition of PGI_2 synthetase by the variety of hydroperoxides suggested to these workers the generation of a common reactive species rather than a structural interaction. Bourgain (1980) demonstrated that 15-HPAA increases thrombus formation *in vivo*. This hydroperoxide-mediated inhibition of PGI_2 synthetase may have important consequences in atherosclerosis and explain why these vessels produce less PGI_2 (Higgs *et al* 1980).

PGI_2 production also decreases with age (Kent *et al* 1981). Lipid peroxidation can take place in plasma as a nonenzymatic reaction in the presence of high concentrations of molecular oxygen with metal ions as catalysts (Harman and Piette 1966) and lipid peroxides are formed in pathological conditions (Slater 1972). Szczeklik and Gryglewski (1980) have shown that serum lipid peroxides are concentrated in the low density lipoprotein fraction (LDL) and that LDL can decrease PGI_2 production by rat aorta. This may be related to the increase in PGI_2 production seen in rats treated with hypolipidemic drugs (Weithman and Granzer 1980). Greenwald *et al* (1980) have shown that blood vessels themselves, produce lipoxygenase products.

The current interest in selecting suitable conditions in order to obtain specific platelet cyclo-oxygenase inhibition over blood vessel cyclo-oxygenase inhibition with ASA led us to test the present series of ASA analogs on the PGI_2 synthesis system. Although animal tissue was studied, valuable information could be obtained by comparing the rank order of potencies of these agents, which are structural analogs of ASA but exhibit a wide range of activities on platelets and PGs. In light of what is already known of these agents in platelet systems and the current interest in hydroperoxides, these agents were assayed using a rapid and sensitive bioassay system.

2.4.1.2 **Methods**

Measurement of prostacyclin activity by human platelet aggregation bioassay

Prostacyclin sodium salt (PGI_2Na^+) (Upjohn Co., Kalamazoo, Michigan) was tested on ADP-induced platelet aggregation in PRP from a number of donors. PGI_2Na^+ was made up as a lug/ml solution in Tris-HCl buffer (0.05M, pH 9.0) and stored on ice. ADP-induced platelet aggregation using a Born aggregometer was performed as described above. PGI_2Na^+ (10ul), which was diluted to the appropriate stock solution in Tris-HCl buffer (0.05M, pH 7.4) immediately before use, was added to the PRP which had incubated 3 minutes and this was stirred for 1 minute further at which time the ADP was added. All curves were compared to control curves in which only similarly prepared Tris buffer was added.

Bioassay of drug effects on PGI_2 production by rabbit aorta rings

New Zealand white rabbits (2-3kg) were anesthetized with sodium pentobarbital (Nembutal) 60mg/kg by intravenous infusion using a Butterfly-21 infusion set (Abbott) connected to a 5ml glass syringe. Infusion took place through the marginal vein of the ear.

A midline incision was made and blood was removed through the inferior vena cava with a 19 gauge needle and 50ml disposable plastic syringe. The rib cage was opened and the thoracic aorta was carefully removed and placed in ice cold Tris-HCl buffer (0.05M pH 7.4) where it was cleared of excess tissue and cut into rings. The ring weight was 33 $\pm$ 6mg wet weight. The rings were transferred to a 10ml beaker of ice cold Tris for storage and all rings were used within 3 hours.

"Exhausted" rings were prepared by incubating fresh rings in Tris buffer at room temperature with frequent changes of buffer, for at least 1 hour. A ring was considered exhausted when it ceased to produce platelet inhibition activity.

Each ring was incubated for 6 minutes at room temperature in 100ul of Tris-HCl buffer (0.05M pH 7.4) or drug dissolved in Tris buffer, in Eppendorf Microtest tubes. At this time the ring in Tris was vortexed briefly and 20ul of supernatant was transferred to a cuvette containing 350ul of human PRP which had been incubating for 3 minutes at 37° C. The PRP was stirred for 1 minute in a Born aggregometer at 37° C and then ADP was added and aggregation was measured (as in AA aggregation) (Fig. 14). The inhibition of PGI_2-production was determined from the 5 minute maximum curve height of 3 curves run in a series: 1. ring in Tris 2. ring in drug and 3. exhausted ring in drug (Fig. 16A). Under these conditions only the highest drug concentration had effects on platelet aggregation but these effects were minimal because of the dilution factors and short incubation times.

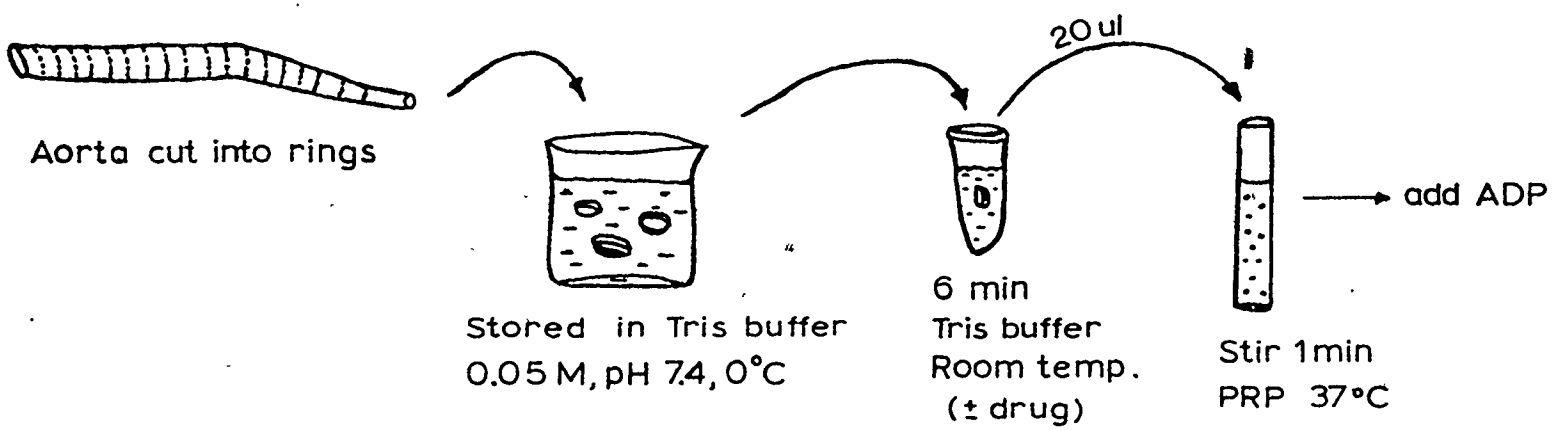
In some studies PGI_2-like activity production by rat aorta was also assayed by a similar procedure (see also 2.5.1.2).

Assay for antagonism of PGI_2 activity

To test for possible antagonist effects of the agents studied on the PGI_2 receptor, drugs were added to PRP, at the highest concentration attained in the bioassay, immediately prior to a submaximal inhibitory dose of PGI_2Na^+. Aggregation was then carried out as described above for PGI_2Na^+. Aggregation curves from PRP in the presence of PGI_2Na^+ and drug together were compared to those containing each agent alone.

Fig. 14 Technique for bioassay of PGI_2-like activity from aorta rings.

Fresh aorta was removed from animals, cut into rings and stored in Tris buffer (0.05 M pH 7.4) on ice. To bioassay PGI_2-like activity, one or more rings were incubated together in Tris buffer, either with or without drug, for 6 min at room temperature. A portion of the supernatant was then added to a cuvette of PRP, preincubated at 37° C and the mixture stirred for 1 min in a platelet aggregometer before the addition of the platelet aggregating agent (ADP).


Aorta cut into rings
Stored in Tris buffer
0.05 M, pH 7.4, 0°C
20 ul
6 min
Tris buffer
Room temp.
(± drug)
Stir 1 min
PRP 37°C
add ADP

2.4.1.3 **Results**

An estimate of the dose-effect profile of PGI_2Na^+ on human PRP ADP aggregation was obtained by constructing a dose-effect curve from the data pooled from 4 different blood donors (Fig. 15, 16B). The 5 minute total height of ADP aggregation curves was inhibited in a dose-related fashion with an ID_{50} of about 3pmol/ml. No inhibition occurred at concentrations less than 1pmol/ml and aggregation was totally inhibited at 20pmol/ml. The first phase of aggregation was less sensitive to PGI_2Na^+ with an ID_{50} value close to 4pmol/ml.

The dose dependent effect of PGI_2-like activity from rat aorta on rat heparinized PRP is shown in Fig. 16C. The time course of PGI_2 production by rat aorta in Tris-HCl buffer (0.05M pH 7.4) on human PRP is shown in Fig. 16D and rabbit aorta on rabbit PRP (Fig. 17A). The pH sensitivity of rabbit aorta produced PGI_2-activity is demonstrated by Fig. 17B. PGI_2-like activity was inhibited by indomethacin (10uM) and tranylcypromine (1mg/ml). All these data are consistent with the chemical stability of PGI_2 (Johnson et al 1976).

All agents were tested for effects on PGI_2-like activity generated by rabbit aorta rings, using a human platelet aggregation bioassay. The effects of ASA and 2-PBA (Fig. 17C, 18) were indistinguishable in this system. ABA caused weak inhibition at low doses but it lacked a concentration dependent inhibitory effect while 3-PBA caused potentiation of PGI_2-like effects over the concentration range tested. 3-MP (Fig. 18B) had an inhibitory effect on PGI_2 production but the results were variable and the inhibition was not dose related. 3HMP was the most potent inhibitor of PGI_2-like activity. The effects were

Fig. 15 Dose-response effects of PGI_2Na^+ on ADP-induced platelet aggregation.

% Inhibition of A. first phase height and B. 5 min total height of ADP-induced aggregation versus the logarithm of the concentration of PGI_2Na^+ in ng/380 ul final volume. PGI_2Na^+ was made up to the final concentration in Tris (0.05 M pH 7.4) immediately before addition to PRP and was added to PRP 1 min before the addition of ADP (4.5 uM). Dose-response curves were estimated from the pooled data from 4 different people (each marked by a different symbol) who exhibited biphasic aggregation to 4.5 uM ADP. ID_{50} for PGI_2 inhibition of first phase height was 4 pmol/ml; total height 3 pmol/ml. PGI_2Na^+ MW. 374.45.

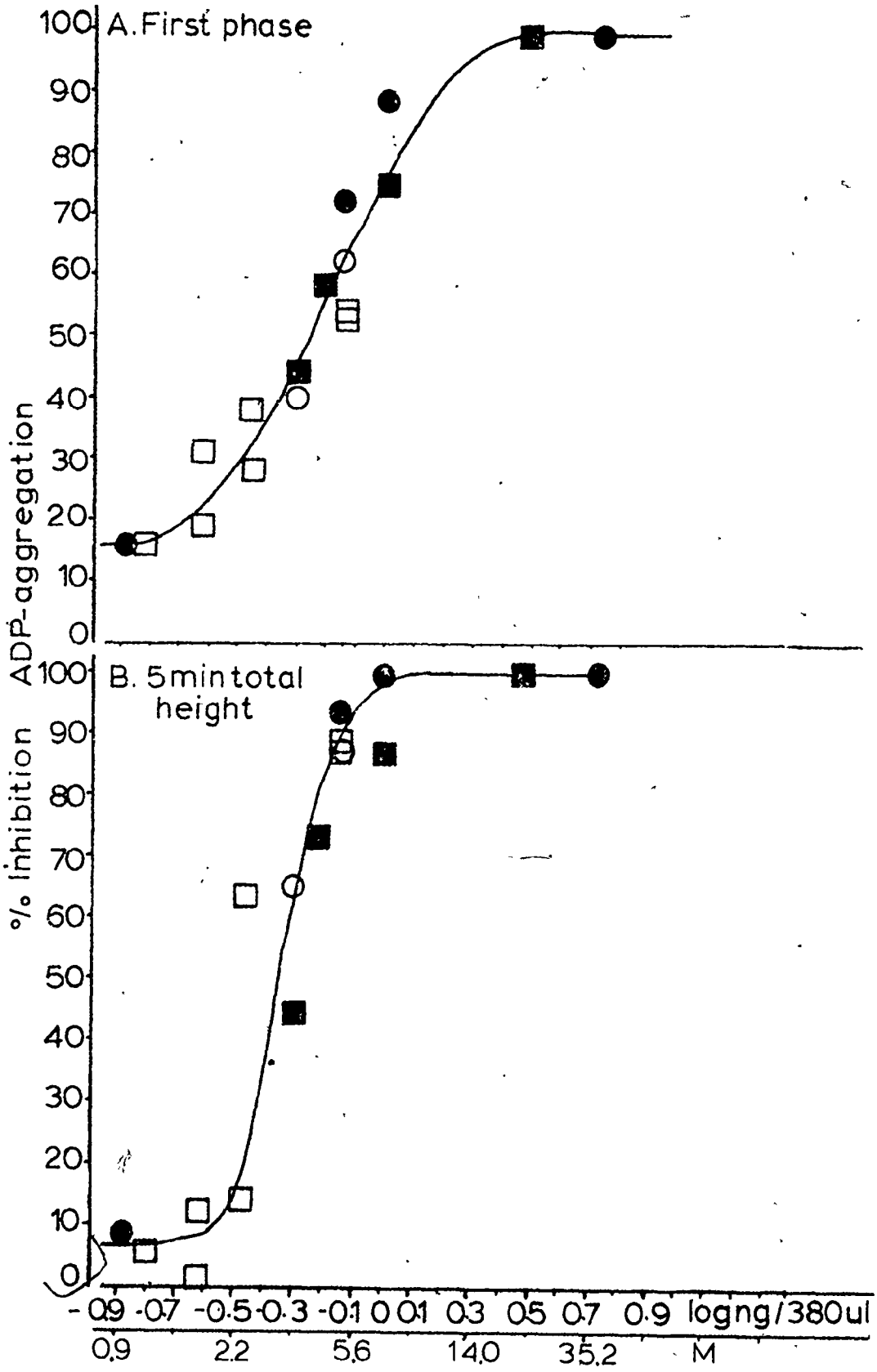

A. First phase
B. 5 min total height
% Inhibition ADP-aggregation
100
90
80
70
60
50
40
30
20
10
0
-0.9 -0.7 -0.5 -0.3 -0.1 0 0.1 0.3 0.5 0.7 0.9 log ng/380ul
0.9
2.2
5.6
14.0
35.2
M

Fig. 16 The effects of PGI_2Na^+ and PGI_2-like activity from aorta on platelet aggregation.

A. Formula for the calculation of % inhibition of PGI_2-like activity by benzoic acid analogs. Fresh aorta ring(s) were incubated in buffer containing test drug and the PGI_2-like activity released into the buffer, as assayed on human platelet aggregation, was compared to the PGI_2-like activity from fresh ring(s) in buffer alone and "exhausted" ring(s) in buffer containing the test drug.

B. Typical effects of PGI_2Na^+ on human platelet aggregation. PGI_2NA^+ was made up to the final concentration in Tris (0.05 M pH 7.4) immediately before addition to PRP and was mixed with PRP (37° C) 1 min before the addition of ADP (4.5 uM).

C. Typical effects of PGI_2-like activity from rat aorta rings on rat PRP anticoagulated with heparin. Rat PRP was aggregated with ADP (4.5 uM).

D. Typical effects of time on PGI_2-like activity from rat aorta rings. A ring was incubated in Tris buffer (0.05 M pH 7.4) and assayed at 3, 12 and 20 min for PGI_2-like activity. Human platelet aggregation inhibition was used as the bioassay and aggregation was induced with ADP (4.5 uM).

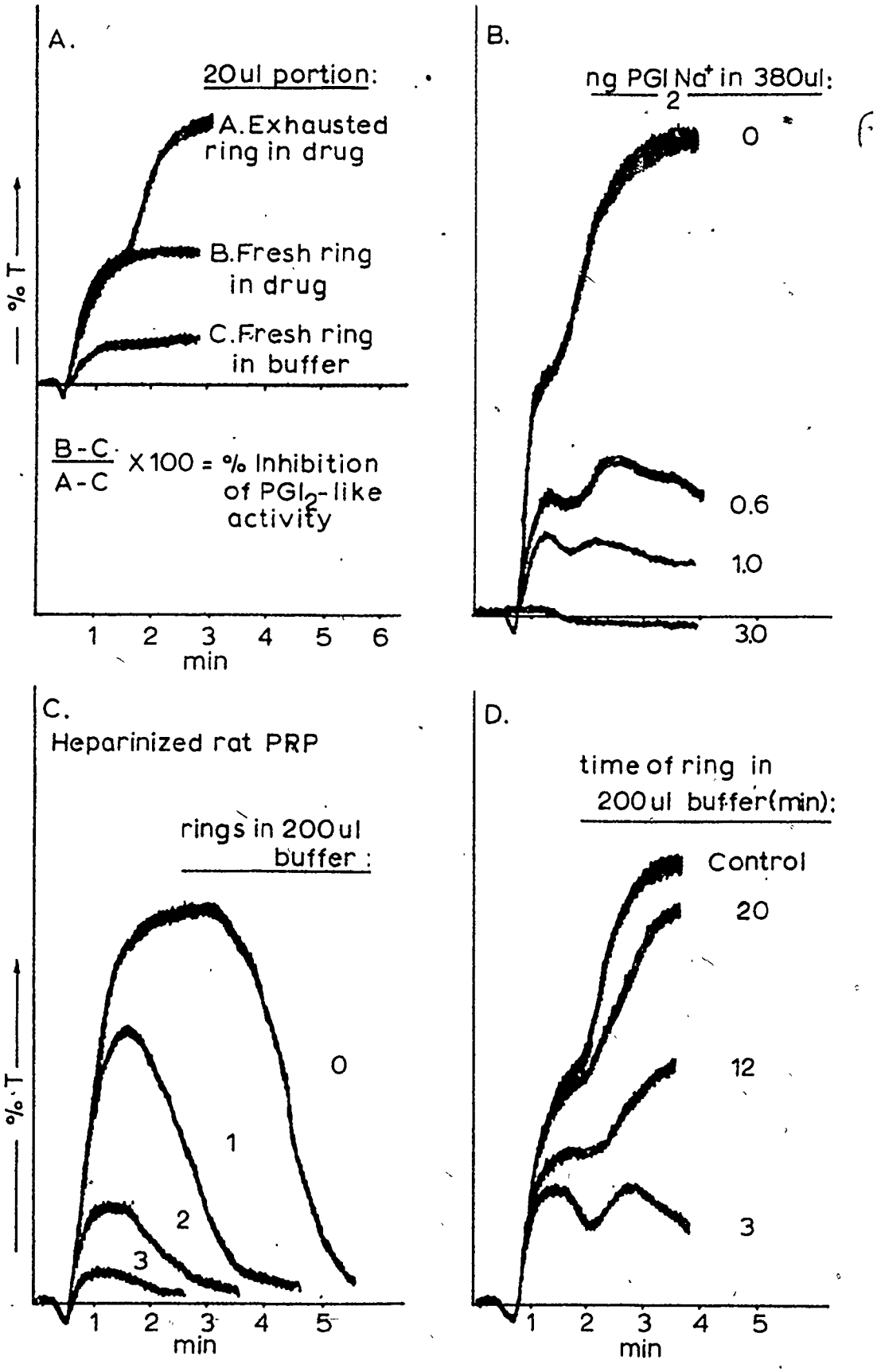

A.
20ul portion:
A. Exhausted ring in drug
B. Fresh ring in drug
C. Fresh ring in buffer
% T
B-C / A-C X 100 = % Inhibition of PGI2-like activity
1 2 3 4 5 6
min
B.
ng PGI2 Na+ in 380ul:
0
0.6
1.0
3.0
C.
Heparinized rat PRP
rings in 200ul buffer:
0
1
2
3
% T
1 2 3 4 5
min
D.
time of ring in 200ul buffer(min):
Control
20
12
3
1 2 3 4 5
min

Fig. 17 Typical effects of time, pH and ASA on PGI_2-like activity produced by aorta rings.

A. Rabbit aorta rings were incubated in Tris buffer (0.05 M pH 7.4) for various times before the transfer of 20 ul of supernatant to citrated rabbit PRP at 37° C. ADP (10 uM) was added 1 min after the addition of supernatant.

B. Rabbit aorta rings were incubated in Tris buffer (0.05 M pH as indicated) for 6 min before the transfer of 20 ul of supernatant to human PRP at 37° C. ADP (4.5 uM) was added 1 min after the addition of supernatant.

C. Rabbit aorta rings were incubated in Tris buffer (0.05 M pH 7.4) containing various concentrations of ASA for 6 min before the transfer of 20 ul of supernatant to human PRP at 37° C. ADP (4.5 uM) was added 1 min after the addition of supernatant. The "exhausted ring" had been depleted of PGI_2-like activity.

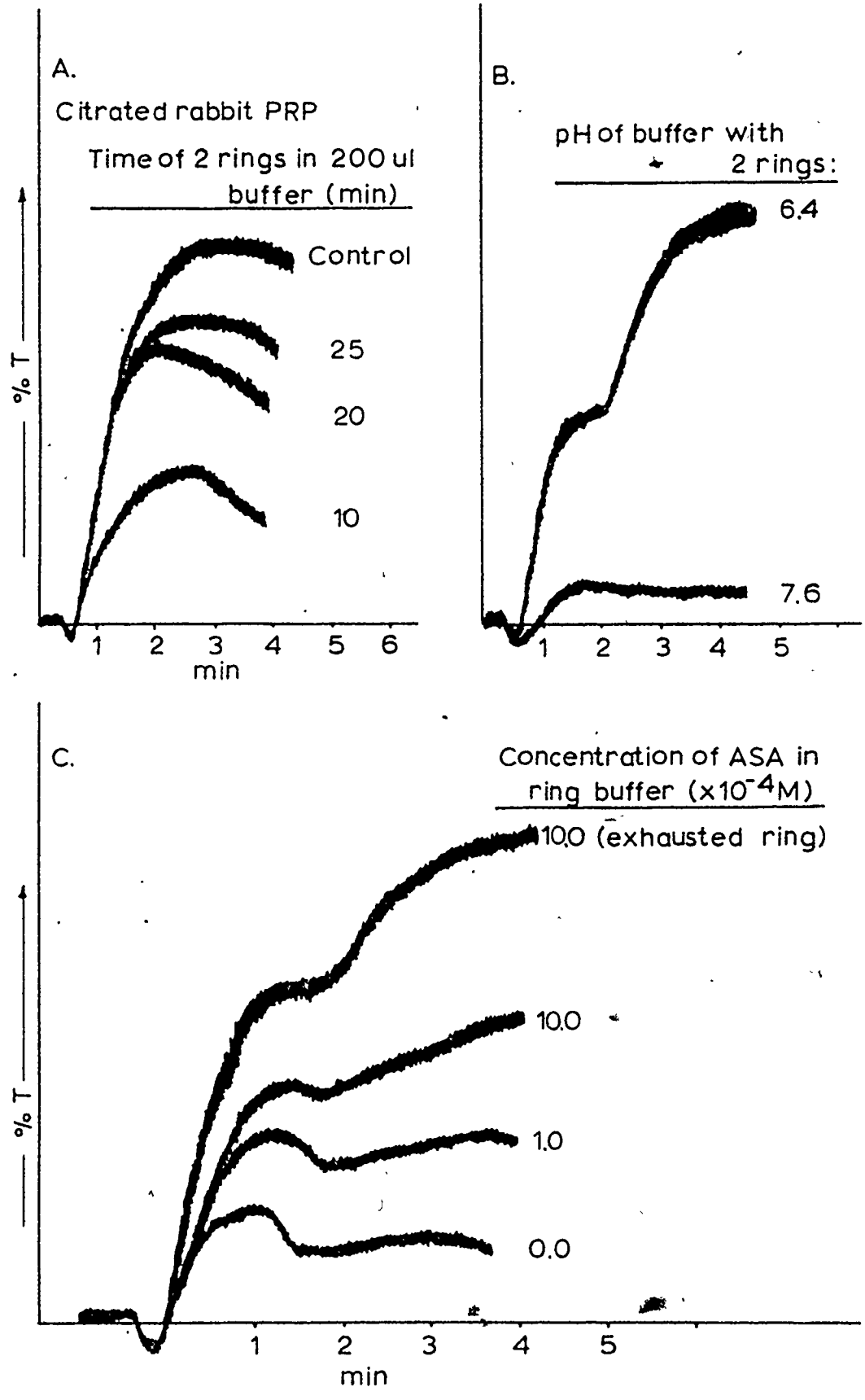
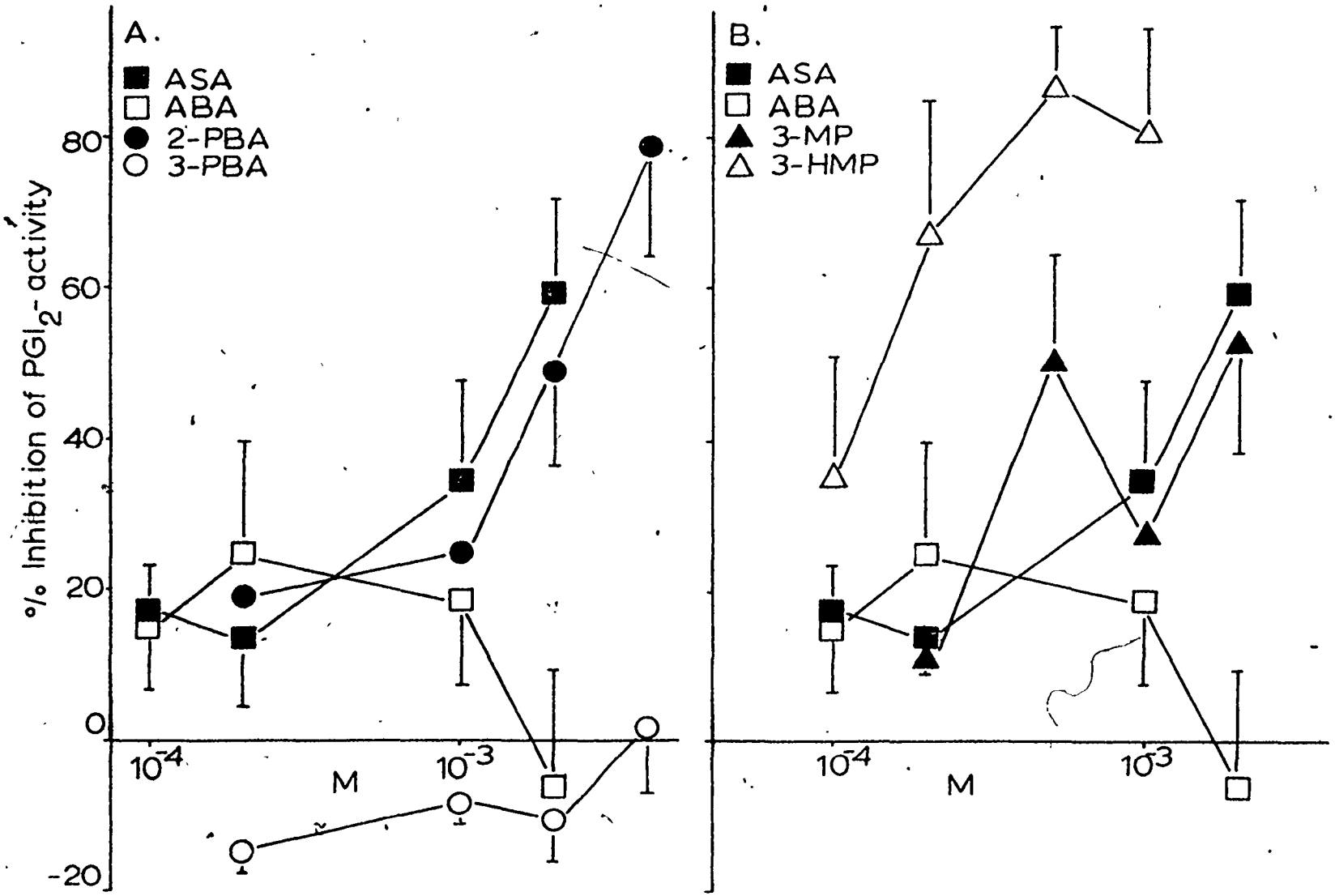

A.
Citrated rabbit PRP
Time of 2 rings in 200 ul buffer (min)
Control
25
20
10
% T
1 2 3 4 5 6
min
B.
pH of buffer with 2 rings:
6.4
7.6
1 2 3 4 5
C.
Concentration of ASA in ring buffer (x10⁻⁴M)
10.0 (exhausted ring)
10.0
1.0
0.0
% T
1 2 3 4 5
min

Fig. 18 Dose-response effects of benzoic acid analogs on PGI_2-like activity from rabbit aorta rings as measured by human platelet aggregation bioassay.

Fresh rings of rabbit aorta were incubated in buffer containing various concentrations of benzoic acid analogs and the % inhibition of PGI_2-like activity was calculated as in Fig. 16A. Each point represents the mean of 6 determinations ± standard error of the mean. All PRP used in this assay exhibited biphasic platelet aggregation at about 4.5 uM ADP.

A. ASA and other benzoic acid analogs existing in the chain configuration: ABA, 2-PBA, 3-PBA.

B. ASA and other benzoic acid analogs existing in the ring configuration: ABA, 3-MP, 3HMP.


A.
ASA
ABA
2-PBA
3-PBA
% Inhibition of PGI_2-activity
80
60
40
20
0
-20
10^{-4}
M
10^{-3}
B.
ASA
ABA
3-MP
3-HMP
10^{-4}
M
10^{-3}

dose-dependent and displayed the same steep dose-response curve as seen in platelet aggregation studies.

A brief study was undertaken to examine these agents for effects of PGI_2 antagonism. When 3-MP (10^{-4}M) was added to PRP immediately prior to a submaximal inhibitory dose of PGI_2Na^+, there was an increased inhibition of aggregation that appeared greater than the sum of PGI_2Na^+ and 3-MP alone. Some additive inhibition, with no evidence for antagonism, was seen with the other agents.

2.4.1.4 **Discussion**

Although the dose-response curves were prepared from data pooled from different donors, they provide an indication of the sensitivity of ADP-induced human platelet aggregation to PGI_2. The chemical stability of the PGI_2-like activity and the effects of such agents as indomethacin and tranylcypromine on its production provide evidence that this activity is, in fact, due to prostacyclin. Human platelet aggregation in citrated PRP was more sensitive to this activity than rat or rabbit citrated PRP but rat PRP anticoagulated with heparin and in a 1:1 dilution with saline, was also very sensitive.

The benzoic acid analogs displayed basically the same rank order of inhibitory potency here as on platelet PG synthesis. 3-MP was the notable exception. ASA and 2-PBA demonstrated similar dose-response patterns and the increased potency of ASA on PG synthesis was not found on PGI_2-like activity - at least under these assay conditions. ABA had weak inhibitory effects but these were not dose-dependent and 3-PBA appeared to increase PGI_2 activity. This is of interest when considered along with the reports that 3-PBA increased platelet aggregation (Mills

et al 1974), platelet ATP release (see 2.3.1.3) and various other parameters associated with PG production (Cerskus 1978).

3HMP was the most potent inhibitor of PGI_2-like activity. The site of its action, however, is unclear. As reported in the introduction, hydroperoxides, especially lipid peroxides, are known potent inhibitors of the PGI_2 synthetase enzyme. 3HMP was also a potent inhibitor of the cyclo-oxygenase enzyme and Aharony et al (1981) reported that 12-HPETE inhibited platelet cyclo-oxygenase activity. Finally, the effect of this (2.3.1.3) and other hydroperoxides on both phases of ADP-induced platelet aggregation (Porter et al 1976) suggest other sites of action.

The effects of 3-MP appear biphasic and complicated. The very weak effect of this agent on platelet PG synthesis suggested to us that it may also not affect production of PGI_2-like activity. It was however, a potent inhibitor of both first and second phase ADP aggregation and this led us to discover the PDE inhibitory potential of this agent. The recent work of Hopkins and Gorman (1981) demonstrating the sensitive control of cAMP on PGI_2 production and, further, the role of PDE in the control of cAMP, provide us with a possible explanation for these effects. For instance, at low concentrations, 3-MP may preferentially inhibit blood vessel PDE in such PGI_2 producing cells as the endothelium. The elevations in cAMP would account for the decrease of PGI_2 production. As the concentration increases some PGI_2 may still be produced but now the effect of this PGI_2 is potentiated through 3-MP inhibition of platelet PDE. At higher concentrations, complete inhibition of PGI_2 synthesis may occur through combined PDE inhibition and weak PG synthesis inhibition. Alternatively to this hypothesis, membrane stabilizing effects of 3-MP are possible and have not been

examined.

Although none of the agents tested antagonized the action of PGI_2Na^+, the synergistic activity between it and 3-MP provides further evidence for a cyclic nucleotide mediated interaction between these two agents.

From this study we conclude that we are unable to separate antiplatelet PG effects from effects on PGI_2-like activity of rabbit aorta. Further investigation of the mechanism of action of 3-MP, 3HMP, 3-PBA and perhaps even 2-PBA in relation to ASA may be fruitful in understanding the similarities and differences between these enzymes.

2.4.2 Effects of Benzoic Acid Analogs on Arachidonic Acid Metabolism by Aorta Rings

2.4.2.1 Introduction

Bunting et al (1976) reported that although aorta tissue converted only small amounts of AA to PGI_2, large amounts of PGH_2 were converted to PGI_2. In contrast to this report, Needleman et al (1978) and Baenziger et al (1979) demonstrated good ^{14}C-AA conversion by vascular tissues and Needleman suggested that it was the preferred substrate for PGI_2 synthesis. Herman et al (1979) reported low conversion of AA by rabbit aorta while Panganamala et al (1980, 1981) reported that rat aorta tissue converted 10-15% of the ^{14}C-AA to 6-keto $PGF_{1\alpha}$. The differences in the reports of the amount of ^{14}C-AA converted to PGI_2 may depend on some or all of: the age of the tissue (animal), the species and the substrate concentration.

In the following discussion, PGI_2 and its stable breakdown product 6-keto $PGF_{1\alpha}$, are used interchangeably to represent PGI_2 production.

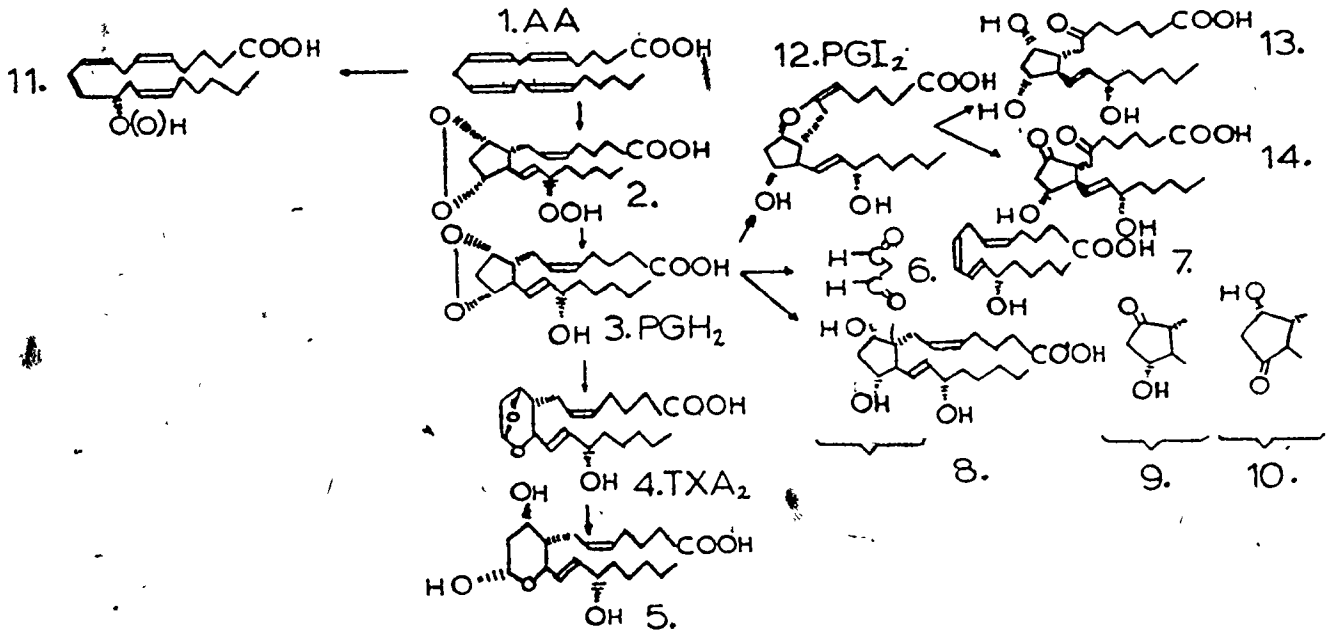
Bunting et al (1976) reported that PGI_2 was the major PG produced by the aorta tissue and Salmon et al (1978) reported only a low concentration of endoperoxide isomerase in vascular tissue. In contrast to these reports, a number of studies have suggested that PGI_2 is only one of a number of metabolites formed from various vascular tissues and vascular cell preparations and, although PGI_2 may be the major metabolite, others may be formed in significant quantities. Terragno and Terragno (1979) have suggested that PGE_2 plays an important role and

Wong et al (1981) have demonstrated that PGI_2 may be transformed to 6-keto PGE_1. Raz et al (1977) and Baenziger et al (1979) reported large levels of both 6-keto $PGF_{1\alpha}$ and PGE_2 in bovine coronary artery and in various vascular cells. Ingerman-Wojenski et al (1981a) reported that endothelial cells produced 6-keto $PGF_{1\alpha}$, TXB_2, PGE_2 and $PGF_{2\alpha}$ when incubated with ^{14}C-AA while smooth muscle cells produced only 6-keto $PGF_{1\alpha}$. Salzman et al (1980) reported that rabbit pulmonary artery produced TXB_2 and possibly 6-keto PGE_1. Claeys et al (1981) reported that chicken aorta produced large amounts of PGE_2 and no PGI_2 at all. Parks et al (1981a) and Whorton et al (1981) reported that pig aorta cells produced metabolites other than PGI_2, including PGE_2. Johnson et al (1981) reported that pulmonary endothelial cells in culture produce 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$ and PGE_2 and Peskar et al (1980) reported that rat vascular tissue metabolized PGI_2 to 6,15-diketo $PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro $PGF_{1\alpha}$. Powell (1981) reported that other metabolites besides PGI_2 arose from the incubation of ^{14}C-AA with fetal calf aorta fractions and they included 6,15-dioxo $PGF_{1\alpha}$, which arose via a hydroperoxy precursor, and 11- and 15-monohydroxyacids, which were suggested as regulators of PG biosynthesis. Herman et al (1979) and Greenwald et al (1980) also reported 12-HETE production by rabbit aorta and suggested a role for this compound as a regulator of PG synthesis. The major products of AA metabolism by blood vessels are demonstrated in Fig. 19.

It is now generally accepted that cyclo-oxygenase activity exists in vascular tissue cells. An active area of research in the control of thrombosis involves the specific inhibition of the platelet enzyme over the vascular enzyme. Apart from differences in cell enzyme synthesis

Fig. 19 The major cyclo-oxygenase and lipoxygenase products of blood vessel AA metabolism.

1. Arachidonic acid (AA)
2. Prostaglandin G_2 (PGG_2)
3. PGH_2
4. Thromboxane A_2 (TXA_2)
5. TXB_2
6. Malondialdehyde (MDA)
7. 12-hydroxyheptadecatrienoic acid (HHT)
8. $PGF_{2\alpha}$
9. PGE_2
10. PGD_2
11. 12-hydroxy (hydroperoxy) eicosatetraenoic acid (12-H(P)ETE)
12. Prostacyclin (PGI_2)
13. 6-keto-$PGF_{1\alpha}$
14. 6-keto-PGE_1


11.
COOH
O(O)H
1.AA
COOH
COOH
OOH 2.
COOH
OH 3.PGH2
COOH
OH 4.TXA2
OH
HO
COOH
OH 5.
12.PGI2
COOH
HO
OH
OH
13.
HO
COOH
14.
HO
OH
COOH
OH 7.
H
H
6.
HO
COOH
OH
OH
8.
OH
9.
HO
O
10.

capabilities, the controversy over the sensitivity of vascular cyclo-oxygenase relative to platelet cyclo-oxygenase is summed up by the reports of Baenziger et al (1979) who found the vessel wall enzymes to be 14-44 times less sensitive than the platelet enzyme to ASA while Jaffe and Weksler (1979) have suggested similar sensitivities of the two systems (see 2.4.1.1).

Here we examine the sensitivity of rat and rabbit aorta cyclo-oxygenase to inhibition by high doses of the benzoic acid analogs.

2.4.2.2 **Methods**

The conversion of ^{14}C-AA to PGs by rat and rabbit aorta was studied by the method described in section 2.3.2.2 with modifications of the incubation time and substrate concentration.

Male, Sprague-Dawley rats, 250-300g in weight were anesthetized with an intraperitoneal injection of urethane (ethyl carbamate, BDH, Toronto) 6ml/kg of a 25% solution. Fresh, intact rings of rat aorta (15 $\pm$ 5mg) or rabbit aorta (33 $\pm$ 6mg) were prepared as described in section 2.4.1.2. Rings of aorta tissue were incubated at room temperature in Tris-HCl buffer (0.05M pH 7.4) $\pm$ drug for 7 minutes at which time ^{14}C-AA, 10-40uM, was added. After a gentle vortex, incubation was continued for a futher 6-60 minutes at which time the rings were removed and rinsed. The rinse solution and incubation mixture were combined (500ul total volume) and were subjected to the extraction and PG assay procedures as described for the freeze-thawed platelet preparation (2.3.2.2). The % conversion of ^{14}C-AA to PGs was 3%.

2.4.2.3 **Results**

The effects of these agents on rabbit aorta PG production were assessed in preliminary studies by measuring the conversion of ^{14}C-AA into metabolites extracted and separated into the 6% MeOH/$CHCl_3$ fraction from silicic acid column chromatography. This fraction has been shown to contain PGs and TXBs (2.3.2.3). The effects of ASA, ABA, 2-PBA, 3-MP and 3HMP at $2.0x10^{-3}$M on the conversion of 30uM ^{14}C-AA to cyclo-oxygenase metabolites are shown in Fig. 20A. The incubation time was 60 minutes. The effects of 3→PBA were quite variable and are not shown here. Compared to the control levels of radioactivity, ASA, 2-PBA and 3-MP caused noticeable reductions. The effects of ABA were less dramatic while 3HMP caused a massive increase in radioactivity associated with this fraction.

ASA, 3-PBA and 3HMP were studied at a $2.0x10^{-3}$M final concentration using a 20uM ^{14}C-AA substrate concentration, based on the report of Panganamala et al (1980,1981) that the optimal substrate concentration for ^{14}C-AA conversion in rat tissue was close to 15uM and less efficient conversion took place at concentrations over 35uM. Both ASA and 3-PBA reduced the amount of radioactivity in this fraction while 3HMP caused the same large increase as seen at higher substrate concentrations (Fig. 20B).

This fraction was applied to TLC plates and acetic acid/ethyl acetate solvent mixture (1:99, v:v) (I) was used to develop the plates. The radioactivity of a control sample and one incubated with 3HMP $2.0x10^{-3}$M are shown in Fig. 21A. A peak of radioactivity corresponding to PGI_2 and another peak of activity (PGE_2, TXB_2) are seen in the

Fig.-20 The effects of benzoic acid analogs on ^{14}C-AA metabolism by rabbit aorta rings.

A fresh ring of rabbit aorta (30 mg) was incubated at room temperature, in Tris buffer (0.05 M pH 7.4, containing 2 mM benzoic acid analog) for 7 min before the addition of AA (specific activity 5 uCi/umol). The reaction continued for 1 hour then the ring was removed and the chloroform extract of the acidified supernatant was separated on a silicic acid column. CPM represent the counts in the 6% MeOH/$CHCl_3$ fraction. Each bar represents the average of duplicate determinations.

A. ASA, 2-PBA, ABA, 3-MP and 3HMP compared to a Tris control using an AA concentration of 30 uM. All tissue was from the same animal.

B. ASA, 3-PBA and 3HMP compared to a Tris control using an AA concentration of 20 uM. All tissue was from the same animal.

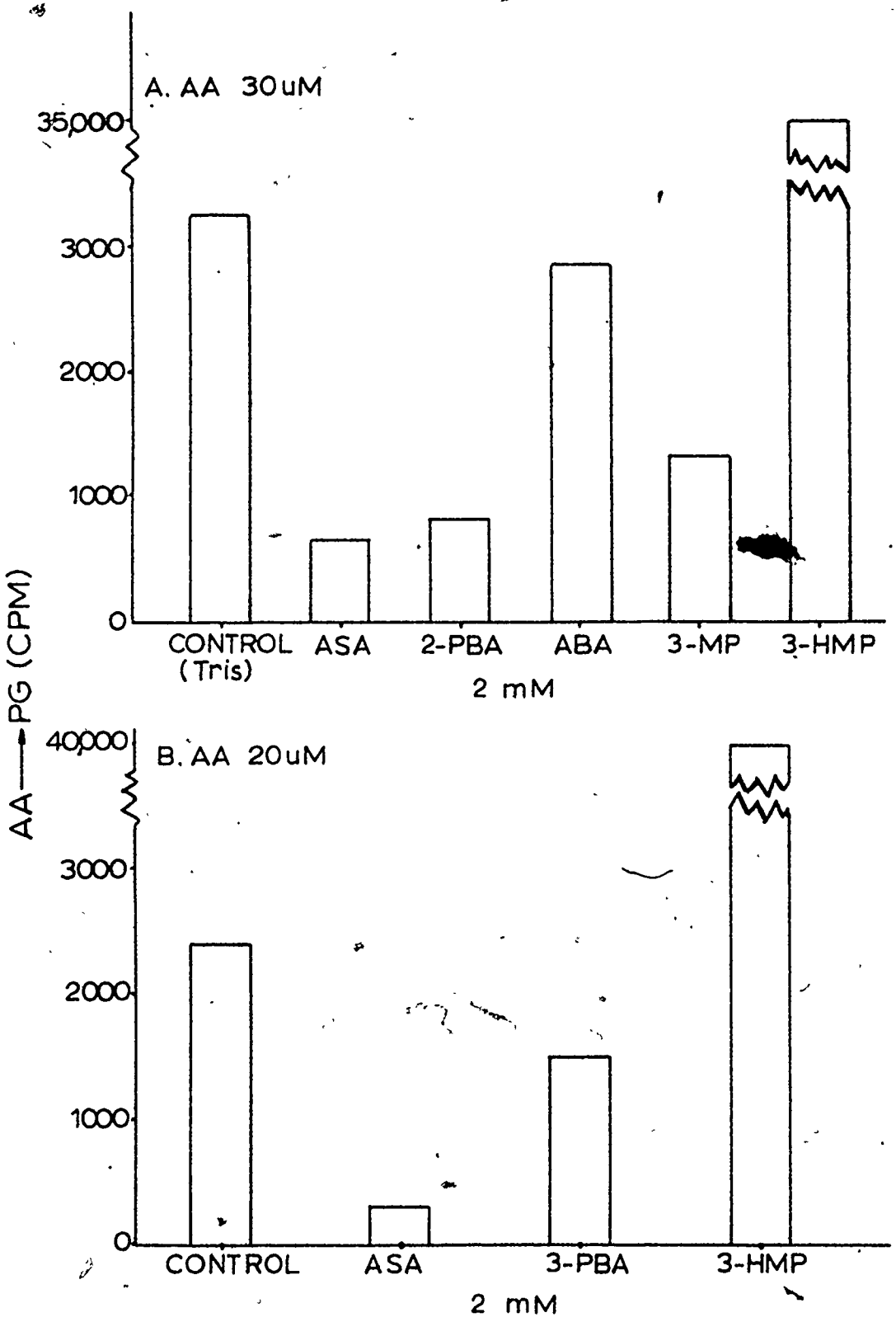

A. AA 30uM
35,000
3000
2000
1000
0
CONTROL (Tris)
ASA
2-PBA
ABA
3-MP
3-HMP
2 mM
AA ⟶ PG (CPM)
B. AA 20uM
40,000
3000
2000
1000
0
CONTROL
ASA
3-PBA
3-HMP
2 mM

Fig. 21 The effects of 3HMP and ASA on the profile of ^{14}C-AA products formed by rabbit aorta rings that co-chromatograph with PGs.

PG standards are indicated. Solvent flows in the direction of the arrow.

A. 3HMP. Methodology as in Fig. 20. The solvent system used here was acetic acid/ethyl acetate (1:99, v:v) with double development. Note the change in scale for the tissue treated with 3HMP.

B. ASA. Methodology as in Fig. 20. The solvent system used here was ethyl acetate/acetic acid/isooctane/water (110:20:50:100, v:v) with double development.

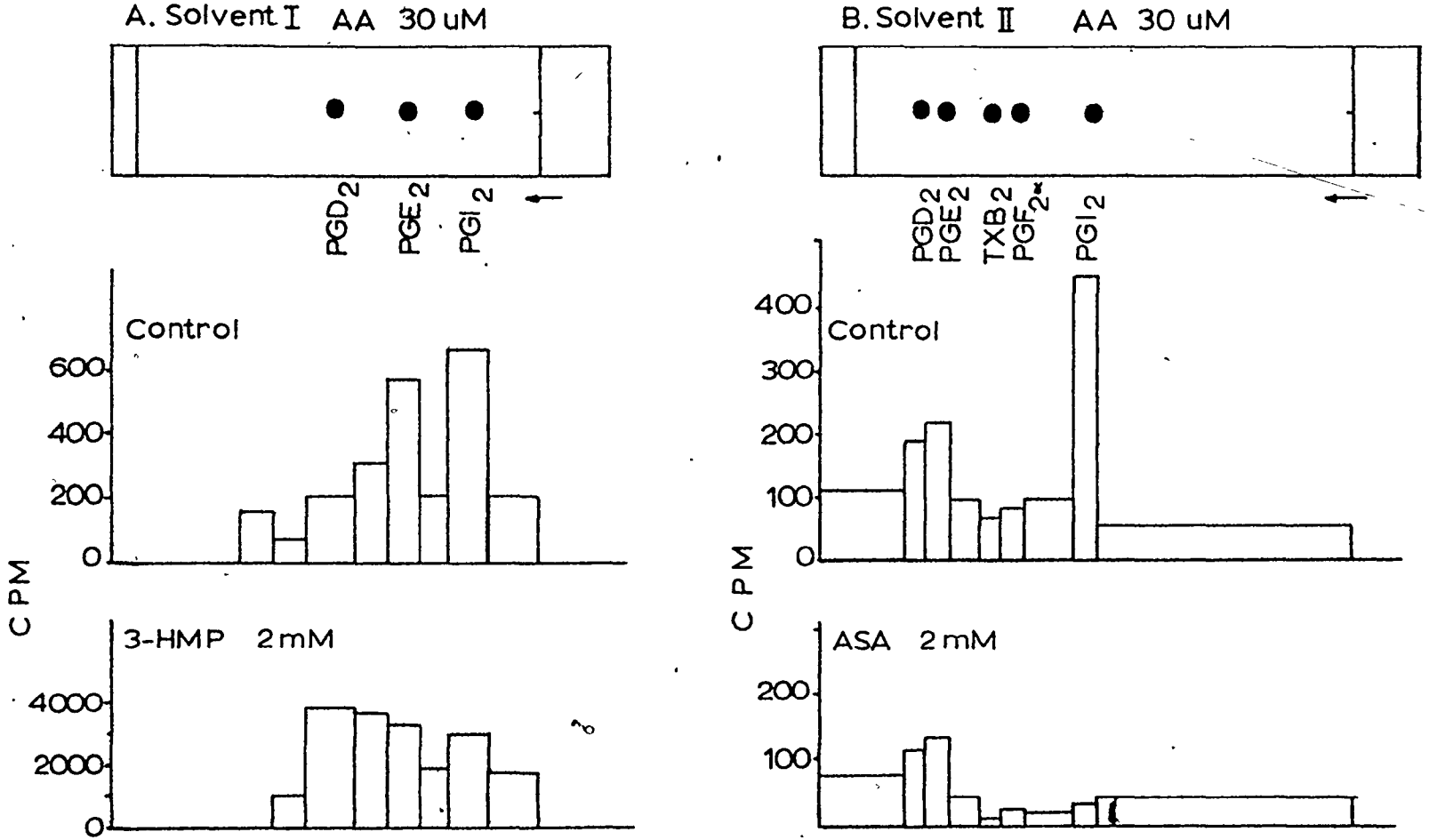
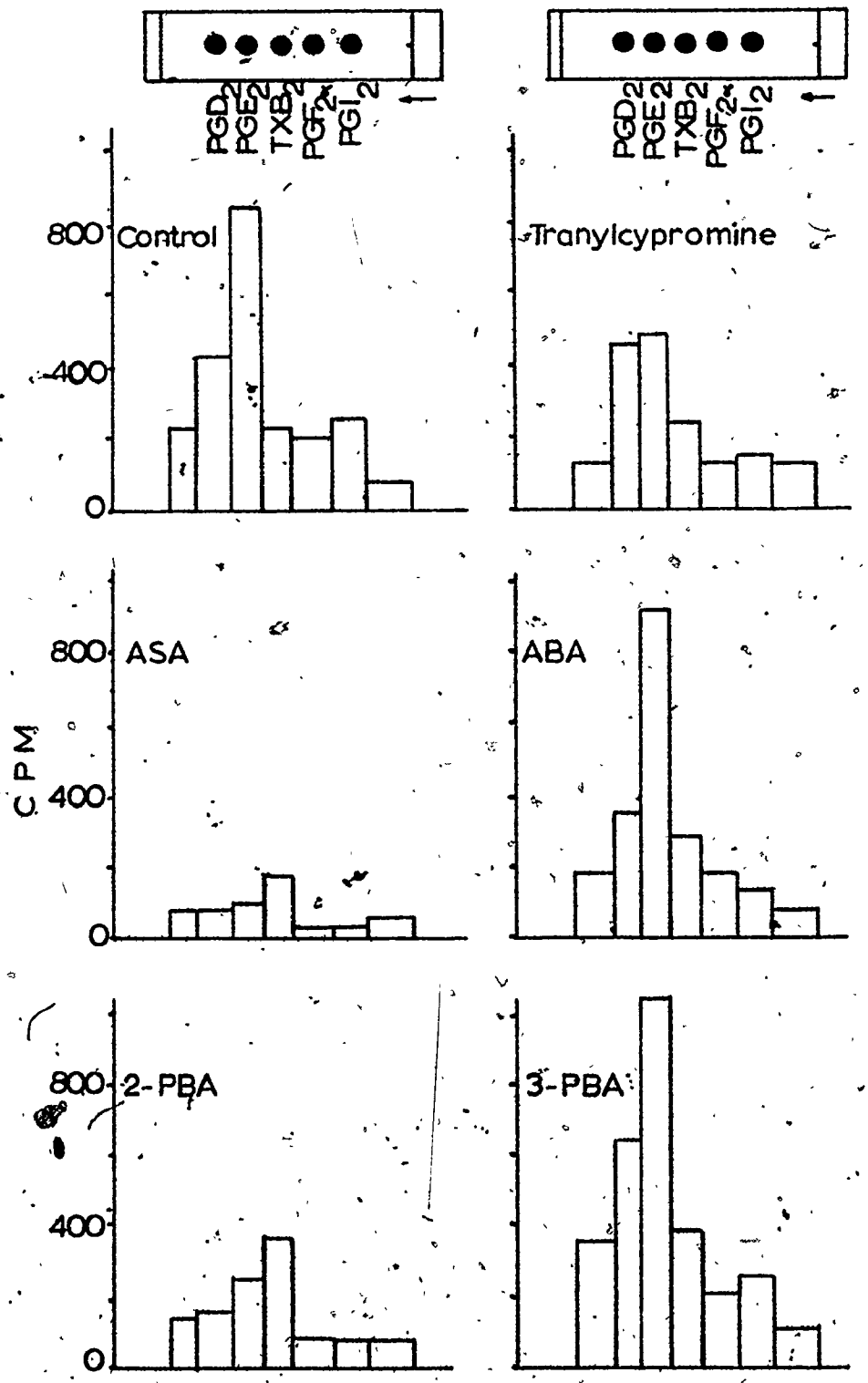

A. Solvent I AA 30 uM
PGD2
PGE2
PGI2
Control
600
400
200
0
CPM
3-HMP 2mM
4000
2000
0
B. Solvent II AA 30 uM
PGD2
PGE2
TXB2
PGF2α
PGI2
Control
400
300
200
100
0
CPM
ASA 2mM
200
100

Fig. 22 The effects of ASA, ABA, 2-PBA and 3-PBA on ^{14}C-AA metabolism by rat aorta rings.

Rat aorta rings (15 mg) were incubated with Tris (0.05 M pH 7.4, containing 2 mM benzoic acid analog) for 7 min before the addition of AA (15 uM) (specific activity 5 uCi/umol). The final reaction volume was 300 ul. The incubation continued for 1 hour then the products were extracted. Products in the 6% MeOH/$CHCl_3$ fraction from silicic acid columns were separated by TLC and compared to known standards. The solvent system was ethyl acetate/acetic acid/isooctane/water (110:20:50:100, v:v) with double development. Benzoic acids were compared to Tris control and tranylcypromine, a prostacyclin synthetase inhibitor. All tissue was from the same animal.


PGD2
PGE2
TXB2
PGF2α
PGI2
Control
Tranylcypromine
ASA
ABA
2-PBA
3-PBA
800
400
0
CPM

control sample. 3HMP caused an apparently nonspecific increase in metabolites over the entire migration area of the PG standards.

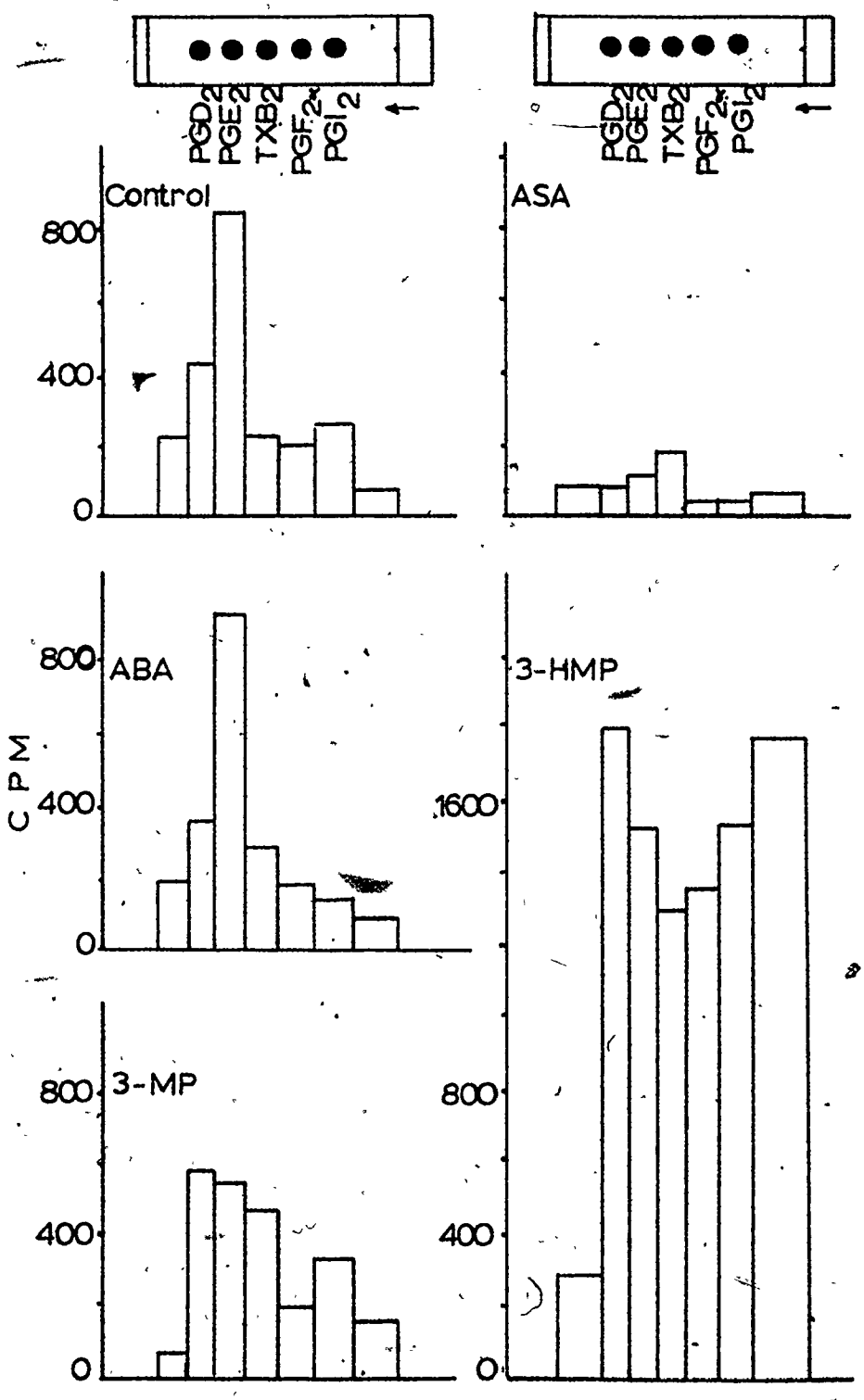
An ASA treated ring was compared to control treatment using this same procedure but using the organic phase of ethyl acetate/ acetic acid/ isooctane/ water (110:20:50:100, v:v) (II) as the solvent system (Fig. 21B). A peak of radioactivity corresponding to PGI_2Na^+ appeared in the control as did small peaks corresponding to PGD_2 and PGE_2. ASA ($2.0x10^{-3}$M) eliminated the PGI_2 activity and reduced the peaks co-chromatographing with PGD_2 and PGE_2.

All agents were tested on ^{14}C-AA metabolism by rat aorta based on the report of Panganamala et al (1980, 1981) that rat aorta converted large amounts of ^{14}C-AA to ^{14}C-PGI_2 compared to rabbit tissue. All agents were tested at 2mM final concentrations and 10uM ^{14}C-AA concentration, using rings from the same animal. The profile of ^{14}C-AA metabolites after developing the plate in solvent II are shown in Fig. 22, 23. The major peaks of radioactivity corresponded to PGE_2 followed by PGD_2, PGI_2 and TXB_2. ASA inhibited all peaks but left a peak of radioactivity at TXB_2. 2-PBA actually caused an increase in radioactivity co-chromatographing with TXB_2 although the other peaks were inhibited. 3-PBA had little effect except perhaps a slight increase at PGE_2 and ABA appeared to cause some inhibition of PGI_2 synthesis (Fig. 22). Both 3-MP and tranylcypromine (Fig. 22, 23) caused reductions in PGE_2 radioactivity but, unlike 3-MP, tranylcypromine also reduced the peak of radioactivity corresponding to PGI_2.

As demonstrated for rabbit tissue, 3HMP increased the radioactivity nonspecifically. This increase was much larger than the 6% MeOH/$CHCl_3$ total radioactivity of a control sample in which 3HMP was incubated with

Fig. 23 The effects of ASA, ABA, 3-MP and 3HMP on ^{14}C-AA metabolism by rat aorta rings.

Procedure as in Fig. 22.


PGD2
PGE2
TXB2
PGF2α
PGI2
Control
ASA
ABA
3-HMP
3-MP
CPM
800
400
0
1600

^{14}C-AA without the addition of an aorta ring (not shown).

The effects of the benzoic acid analogs on ^{14}C-AA metabolism by rat aorta generally reflect their effects on platelet PG synthesis. The results of 3HMP may be explained as a tissue mediated nonspecific oxidation of AA although this is only speculation. The lack of effect of 3-MP on PGI_2 radioactivity was not expected. 3-MP was tested in a bioassay system (2.4.1.2) for effects on rat aorta PGI_2-like activity and at 10^{-3}M 3-MP caused a reversal of this activity.

2.4.2.4 **Discussion**

By incubating aorta rings with AA we bypass such areas of PG synthesis control as the release of AA from the cell membrane stores and, in doing so, we bypass the effects of drugs on this control. This allows a more specific analysis of the enzymes involved in the metabolism of AA - the major site of action of ASA-like drugs on PG synthesis.

The incubation of aorta rings with ^{14}C-AA resulted in the recovery of ^{14}C-AA metabolites in the 6% MeOH/$CHCl_3$ fraction and the biosynthesis of these metabolites was inhibited by ASA. Using this extraction procedure, Ali et al (1977) demonstrated that this fraction contained all the PG and TXB products from platelet incubates. This evidence suggests that in this fraction we are measuring cyclo-oxygenase products. The results of ASA and 2-PBA on the generation of these products by rabbit aorta are not surprising in light of their effects on platelet PG synthesis. 3-MP also had weak antiPG synthesis effects in platelet studies although it was not tested at this high concentration. 3-PBA and ABA had only minor effects despite the high concentrations

used here. In contrast to the potent inhibitory activity of 3HMP on platelet PG synthesis the effect of high concentrations of 3HMP on ^{14}C-AA, in the presence of aorta and with long incubation times, may be a nonspecific one. 3HMP was the most potent inhibitor of PGI_2-like activity in the platelet aggregation bioassay and this suggests that PGI_2 is not one of the metabolites here.

The TLC profile of the 6% MeOH/$CHCl_3$ fraction from rabbit aorta (solvent I) (Fig. 21) demonstrates peaks of radioactivity corresponding to PGI_2 and also PGE_2 and PGD_2. Further evidence for the nonspecific effects of 3HMP is demonstrated by the increase in ^{14}C-AA over the entire length of TLC plate covered by the movement of the PG standards with the solvent movement, instead of corresponding to a specific PG. Further confirmation of PGI_2 production from ^{14}C-AA is shown by the presence of a PGI_2 peak using the second solvent system (II) (Fig. 21). The sensitivity of the peak to ASA is in accord with PGI_2 production sensitivity to cyclo-oxygenase inhibitors. This peak was found to increase in size as the incubation time was increased from 6 to 60 minutes.

Rat aorta was reported to produce high levels of PGI_2 compared to rabbit tissue (Panganamala et al 1981) and, considering the size of rings used, the conversion of ^{14}C-AA by rat aorta is high compared to rabbit tissue in these studies. The major peak of activity did not correspond to PGI_2 although there was a peak which did. Recent evidence suggests that PGI_2 is not the only metabolite of AA formed by blood vessels (see 2.4.2.1) (Baenziger et al 1979, Salzman et al 1980, Claeys et al 1981, Ingerman-Wojenski et al 1981, Johnson et al 1981, Parks et al 1981a, Whorton et al 1981).

The effect of the benzoic acid analogs on the 6% MeOH/$CHCl_3$ fraction from rat aorta followed the known effects of these agents as PG synthesis inhibitors (Fig. 22, 23). ASA and 2-PBA were potent inhibitors but, although not apparent in the bioassay study, ASA demonstrated greater potency than 2-PBA both on rat and rabbit aorta. ABA had some effect on PGI_2 synthesis although the peak was only partially reduced, despite the high drug concentration used. Tranylcypromine caused a reduction in both the PGI_2 peak and the PGE_2 peak and this agrees with the report of Rajtar and de Gaetano (1979) that although tranylcypromine is a PGI_2 synthetase inhibitor, it does affect the cyclo-oxygenase enzyme.

3-MP had no effect on the PGI_2 peak of rat tissue although it did inhibit the PGE_2 activity partially. This effect has been found in repeated experiments. Although 3-MP is a weak PG synthesis inhibitor (Cerskus 1978, 2.3.2.3) it did inhibit PGI_2-like activity in the bioassay and it did reduce the 6% MeOH/$CHCl_3$ radioactivity on rabbit aorta tissue, although not completely. Both these tests were performed using rabbit tissue, therefore we tested 3-MP in a bioassay experiment using rat aorta tissue. The production of PGI_2-like activity was inhibited by 3-MP. It is possible therefore, that 3-MP works through other mechanisms to inhibit the production of PGI_2-like activity and this could include membrane stabilizing effects, inhibition of the release of membrane bound AA or, since 3-MP has been shown to inhibit platelet PDE activity, it may affect PGI_2 activity by elevating cAMP levels in PGI_2 producing cells (Hopkins and Gorman 1981). Rabbit tissue may also be more sensitive to 3-MP under the conditions used here.

Except for the effects of 3HMP, these agents display the same

profile of activity on platelet and aorta PG synthesis. Even where cyclo-oxygenase inhibition is not a factor, the activities could not be separated as 3-MP inhibited both platelet function and the production of PGI_2-like activity although it appears to be a weak cyclo-oxygenase inhibitor. Further study of the nature of the effects of 3-MP and 3HMP may uncover other mechanisms of PGI_2 control and mechanisms of hydroperoxide interaction with PG synthetic enzymes.

The purpose of this study was to investigate the differential sensitivity of aorta cyclo-oxygenase to platelet cyclo-oxygenase not by determining the concentration range required for inhibition by a single agent but by evaluating the rank order of potency of a series of ASA-like drugs both with and without antiPG effects on the platelet. We conclude that, with the exception of 3HMP, the compounds affect cyclo-oxygenase activity in both systems in a similar manner and separation of the effects on one cyclo-oxygenase from the other is not possible with this series of agents. The effects of 3HMP remain unresolved.

The assay of blood vessel capacity to convert AA to PGs may also prove useful in the study of vessels in various disease states and in thrombosis models.

2.5 In Vivo Effects of Benzoic Acid Analogs on Thrombosis

2.5.1 Effects of Benzoic Acid Analogs on the Rat Thrombosis Model

2.5.1.1 Introduction

Initial enthusiasm over the use of the Born Aggregometer as an *in vitro* screening method for antithrombotic drugs was partially dampened by the finding that many other drugs interferred with platelet function in this test but did not always demonstrate antithrombotic activity *in vivo* (Philp 1981). In recent studies the work of Nunn (1981) suggested that ASA had less potent antithrombotic effects in whole blood than PRP and Weiss *et al* (1981) demonstrated that ASA was less active in native blood than citrated whole blood. Furthermore, ASA was generally a weak inhibitor of thrombus formation in samples of human blood suggesting that drug effects in PRP did not necessarily reflect the *in vivo* situation.

Various models of *in vivo* thrombus formation have been proposed and these have been reviewed by Henry (1962), Didisheim (1972), Mustard and Packham (1978), Philp (1979) and Philp (1981). The establishment of a model for a disease state which has a protracted natural history and/or multifactorial, poorly understood etiology, is a complex task and explains why few current models bear close resemblance to the clinical disorders they are designed to mimic (Philp 1981). Thrombosis has been induced experimentally *in vivo* by chemical, surgical, mechanical and other methods including laser injury and arteriovenous shunt formation

using thrombogenic surfaces. The use of electrical injury to induce thrombus formation has been reviewed by Didisheim (1972) and Philp (1979). In small vessels the injury depends on both the amperage and time but not the polarity of the current (Duval et al 1970). While factors such as variations in vessel diameter and drug-induced vasodilation and changes in blood flow cannot be easily controlled, this method provides a means for precise quantitation of the injurious stimulus. Didisheim reported in 1968 that, in general, larger vessels (225-235u) remained occluded with thrombus for shorter periods of time than smaller vessels (100-200u) but mural thrombi and embolization lasted longer in the larger vessels. Begent and Born (1970) proposed that the rate of thrombus growth depended upon the opposing effects of the rate of delivery of platelets to the injury site and the shear rate, which would prevent the adherence.

Hladovec (1971) proposed a model involving electrically-induced injury to the rat carotid artery with thrombus formation assessed by the sudden temperature drop of the vessel downstream from the injury. This model allowed participation of the vessel wall in thrombus formation and was relatively easy to perform and quantitate. Thrombus formation developed more slowly in heavier animals with larger blood vessels as reported in other models. Electron microscopy studies (Philp et al 1978) showed that the electrical current caused extensive damage to endothelial and subendothelial structures. Both granulated and degranulated platelets, as well as damaged fragments of the blood vessel, were found in the thrombotic material.

Bourgain and Six (1974) reported an electrical injury model of thrombosis in which small blood vessels were bathed in an ADP solution.

Thrombus generation was blocked by cyclo-oxygenase inhibitors, and in this model PGI_2 synthetase inhibitors potentiated white thrombus formation (Bourgain 1978).

The discovery of the antiaggregatory PG, PGI_2 (Bunting et al 1976), which was formed through a cyclo-oxygenase enzyme in the vessel wall (Roth and Siok 1978), led to the theory of the balance between the proaggregatory TXA_2 and the antiaggregatory PGI_2 in the normal control of thrombosis (Higgs et al 1980). Other PGs which modify platelet function and are formed in vivo may also play a role in this balance. These include PGD_2 (Ali et al 1977, Smith et al 1980) and PGE_2 (Smith et al 1980, Bult et al 1981, Claeys et al 1981). The report of Higgs et al (1978), that much higher doses of PGI_2 (higher than the blood vessel produced (Dejana et al 1980)) are required to inhibit adhesion compared to aggregation, led to a re-evaluation of the role of this PG in preventing thrombosis. In recent studies fish oil diets, which contain eicosapentanoic acid in place of the platelet proaggregatory PG precursor, AA, and diets causing essential fatty acid deficiency, depress thrombosis in rats (Hornstra et al 1981, Houtsmiller et al 1981). In the latter study normal collagen-induced aggregation and thrombotic tendency were achieved by the addition of small amounts of the AA precursor linoleic acid to the diet. Columbic acid, which is a stereoisomer of linoleic acid and has all the structural-functional capabilities of linoleic acid except that it is not converted to PGs, did not reverse the depressed thrombosis. Together these studies, and reports of bleeding tendencies associated with deficiencies in cyclo-oxygenase activity (Pareti et al 1979), point to a central role of

PGs in hemostasis. The lack of understanding regarding the importance of each PG along with such reports as the inhibition of lipoxygenase products by ASA (Deykin and Vaillancourt 1981) complicate the picture of the effect of ASA in this process.

Reports of the differential sensitivities of platelet versus blood vessel cyclo-oxygenase (Baenziger et al 1979, Ellis et al 1980, Reyers et al 1980) have proven to be controversial (Jaffe and Weksler 1979, Buchanan et al 1980). It is generally accepted however that there is more rapid recovery of blood vessel PG producing capacity compared to platelets. The time of blood vessel recovery has been reported to be as long as 18 hours after 4mg/kg ASA (Ingerman-Wojenski 1981b) but this differs between arteries and veins (Buchanan et al 1980). While Preston et al (1981a) reported that ASA may have a cumulative effect on venous PGI_2 production, Thiessen et al (1981) reported the paradoxical effect of increased thrombosis with ASA after one dose but not two or more doses. Many other disparities can be found in the literature concerning the effects of ASA on the PG systems related to thrombosis and a general picture of the situation must be developed.

In studies in the rat, Livio et al (1979) reported that, although arteries were less sensitive to ASA than platelets (ID_{50} 25mg/kg vs 3.6mg/kg I.P. respectively), veins were equally as sensitive as platelets (ID_{50} 2.3mg/kg). The effect on platelets lasted longer (96-120 hours) than on either veins (24-48 hours) or arteries (less than 24 hours). No dose of ASA inhibited platelet cyclo-oxygenase without impairing the enzymatic activity in vascular tissues. Reyers et al (1980) reported that 2.5mg/kg ASA partially inhibited platelet and vein cyclo-oxygenase in rats without affecting arterial cyclo-oxygenase,

10mg/kg completely inhibited rat platelet cyclo-oxygenase but had measurable effects on both venous and arterial cyclo-oxygenase and doses greater than 50mg/kg blocked all 3 activities. In man, Massóti et al (1979) reported that 3.2-3.4mg/kg was the ID_{50} for ASA on platelets however the ID_{50} for blood vessel PGI_2 was 4.9mg/kg. The report by Blackwell (1978) that white blood cells may produce PGI_2 during clotting, and the report that ingested ASA did not prevent the formation of 6-keto $PGF_{1\alpha}$ in clotted blood (Viinikka and Vlikorkala 1981) suggests other interesting possibilities for the PGI_2 and ASA interactions in thrombosis.

Various studies of the effects of ASA in animal models of thrombosis have led to a wide range of results. Sheppard (1972) demonstrated that 300 mg/kg ASA, orally, 15 hours before probe damage to the rabbit aorta prevented thrombosis whereas a 300mg total dose did not. Spilker and Balken (1973) showed that 500mg of ASA orally 2 days before electrical injury prevented thrombosis formation whereas 250mg, 15 minutes prior to injury did not. Moschos et al (1973) were unable to demonstrate protection from electrically-induced thrombus growth in dogs with 600mg of ASA (Philp et al 1978).

Philp et al (1978) reported that doses of ASA of 200mg/kg had antithrombotic activity in the rat electrical injury-induced thrombosis model while 100mg/kg did not. Rosenblum and El-Sabbam (1977) had previously shown that 100mg/kg administered 60 minutes before injury was antithrombotic but if administered 30 minutes before, was not protective. These data would suggest a dual mechanism of antithrombotic activity of ASA, one at high doses and one which was time dependent.

Kelton et al (1978a) demonstrated a sex difference in the

antithrombotic activity of ASA (10mg/kg I.V.) in a rabbit venous thrombosis study and this may be related to the reduced effect of ASA in females as reported in human clinical trials of ASA in stroke (Barnett et al 1980). Coppe et al (1981) suggested that human females had higher platelet counts and more aggregable platelets both before and after ASA treatment.

Further paradoxical effects of ASA were reported by Kelton et al (1978b) in which ASA (200mg/kg I.V.) increased the deposition of labelled fibrinogen uptake by damaged aorta while 10mg/kg did not. Wu et al (1981) reported that 30mg/kg ASA orally, blocked thrombus formation while 50mg/kg did not. Both of these studies were performed with rabbits. The possibility of different ASA transformations with the different routes of administration may account for some of these effects.

Evidence against an important role of PGI_2 in thrombus formation, was given by Dejana et al (1980) who demonstrated that high doses of ASA did not increase platelet deposition on rabbit aorta despite complete inhibition of PGI_2. Kobayashi et al (1981) demonstrated that 0.3-1.0g ASA daily for 7 days prior to injury reduced thrombus formation despite a complete inhibition of PGI_2 synthesis. Groves et al (1981) demonstrated the importance of other factors in thrombosis by demonstrating that 400mg/kg ASA I.V. did not block thrombus formation on damaged neointima whereas heparin did.

The role of PGI_2 in hemostasis is suggested in human studies reported by O'Grady and Moncada (1978) in which 0.3g ASA was shown to increase the bleeding time two hours later while 3.9g did not. Amezcua et al (1979) found however, that 24 hours after a 3.9g dose of ASA, the

bleeding time was doubled and it remained so for 1 week. Collagen aggregation was suppressed 2 hours after this dose, in this study.

Huang et al (1981) reported differences between rat and human platelets with respect to sensitivity to AA stimulation. Such differences must be considered when using animal models of thrombosis. Although a potent stimulator of human platelet aggregation in citrated PRP, 1mM AA did not aggregate rat platelets, although it did enhance ADP and collagen-induced aggregation in an indomethacin sensitive manner. In contrast to this, rat platelets in heparinized PRP aggregated to 0.25mM AA. These results suggest that rat platelets were more dependent on calcium than human platelets. Further differences between rat and human platelets were the lack of sensitivity of rat platelets to the agonist adrenaline or the platelet inhibitor PGD_2.

The ASA-like drugs used in this study have been shown to exert a variety of effects on a number of parameters of thrombotic activity. Some agents inhibited both phases of platelet aggregation (3HMP, 3-MP) while some inhibited only second phase (ASA, 2-PBA). ABA was without effect over the concentration range tested and 3-PBA, at some doses, caused an increase in platelet activity. Some agents blocked both platelet and blood vessel PG production (3HMP, ASA and 2-PBA), 3-MP affected only the blood vessel, ABA had weak effects and 3-PBA always showed some trend to an increase in PG production. Finally, 3-MP and, under some conditions 3HMP, inhibited platelet PDE activity. Here we tested these agents in the rat thrombosis model of Hladovec (1971) as modified by Philp et al (1978). Philp et al (1978) demonstrated that this model was most sensitive to heparin, however, sulfinpyrazone, VK744

and ASA all had antithrombotic activity under certain conditions. These agents work through a variety of mechanisms to bring about these effects. An attempt was made to increase the sensitivity of the model further by using the lowest amperage and injury time that would give reproducible thrombus formation.

2.5.1.2 **Methods**

Studies on Rat Platelets and Aorta PGI_2 Production

A series of brief, preliminary experiments was performed to study rat platelet aggregation and aorta PGI_2-like activity and to assess the effects of both exogenous and intravenous administration of ASA and other benzoic acid analogs on these parameters.

Male Sprague-Dawley rats 200-250g in weight were anesthetized with urethane as described in the PGI_2 studies (2.4.2.2). ASA and the other agents were made up in a solution of 50% PEG (Carbowax, PEG-600 Fisher Scientific Co., Toronto) and saline. The femoral vein of the right leg was isolated and the drug or vehicle was injected using a 30 gauge x 1/2 inch needle and a disposable 1ml tuberculin syringe. After 15 minutes the abdomen was opened and blood was carefully removed from the inferior vena cava using a 10ml disposable syringe and a 20 gauge needle. In studies using citrate anticoagulated blood, PRP was prepared as described previously and diluted 1:1 with saline. In studies using heparinized blood, blood was withdrawn from the animals directly into a 10ml syringe containing heparin sodium (porcine intestinal mucosa 1000 USP units/ml Organon Canada Ltd.) to give a final concentration of 10 units/ml of blood. PRP was then prepared as usual. The aorta was removed and prepared as described for the PGI_2-like activity assay (2.4.1.2) and

AA was prepared as described in the AA aggregation of human blood (2.3.1.2). As ASA did not appear to affect rat platelet ADP-aggregation under these conditions, PGI_2-activity was assessed on rat platelets from the same animal.

The Rat Thrombosis Model

The effects of the benzoic acid analogs on the thrombotic process were investigated in a rat thrombosis model first reported by Hladovec (1971) and modified by Philp et al (1978) (Fig. 24). Electron microscope studies demonstrate platelet involvement in this model (Plate 2, Philp et al 1978). Male Sprague-Dawley rats 200-250g in weight were anesthetized as described above. Animals were placed on an operating stage heated through water circulation from a 37° C water bath. An electric light bulb directed on the animal, but away from the area of injury, also helped maintain normal temperature. The trachea was exposed surgically and intubated with PE 200 polyethylene tubing. The right and left carotid arteries were exposed and cleared of surrounding tissue for a distance of 10-15cm using blunt tweezers. A small piece of parafilm was positioned under the vessel to further isolate it and provide insulation for the electrodes and thermister. The right femoral vein was exposed and cleared of surrounding tissue and was the site of drug injection. Thrombosis was induced in the carotid artery by means of an electric current (350 V, DC, 1mA) of 1 minute duration delivered by a "Lesion Producing Device" (Stoetling Co., Chicago) through 2 rigid stainless-steel wire electrodes, hooked at the end to support the vessel. The electrodes, positioned 2mm apart, did not impede the flow of blood in the vessel. Thrombosis was marked by a sudden drop in

Fig. 24 Schematic representation of the rat electrical injury-induced thrombosis model.

A. The arrangement of the provoking electrodes and the thermistor on the surface of the carotid artery.
Therm. = thermistor Art. = artery.
Arrow indicates the direction of blood flow.

B. The electrical injury circuit and measuring circuit.
Ther. = thermometer Rec. = recorder.

(based on Hladovec 1971).

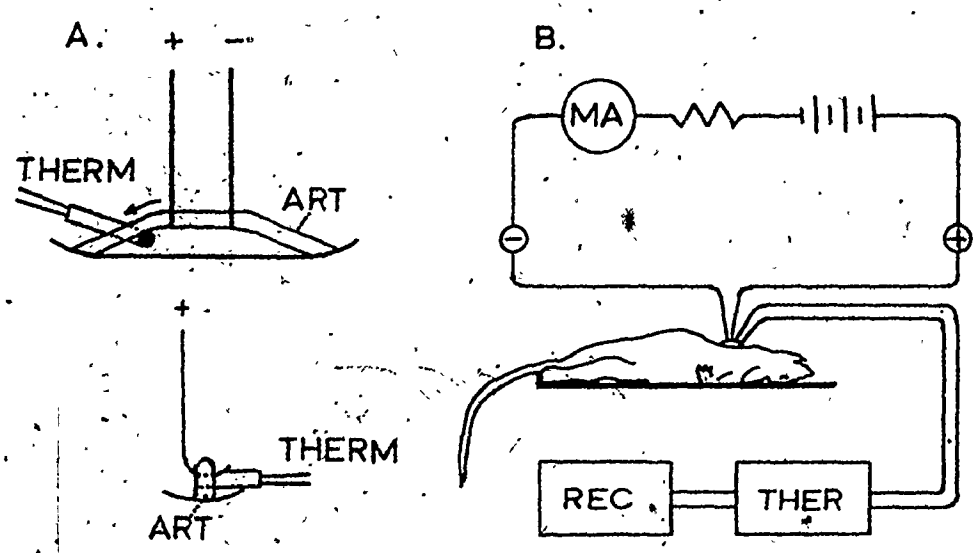

A.
+
—
THERM
ART
+
THERM
ART
B.
MA
REC
THER

Plate 2A Scanning electron micrograph photograph of the luminal surface of the rat carotid artery before injury.
1cm = 5u.

Courtesy of Dr. R.B. Philp

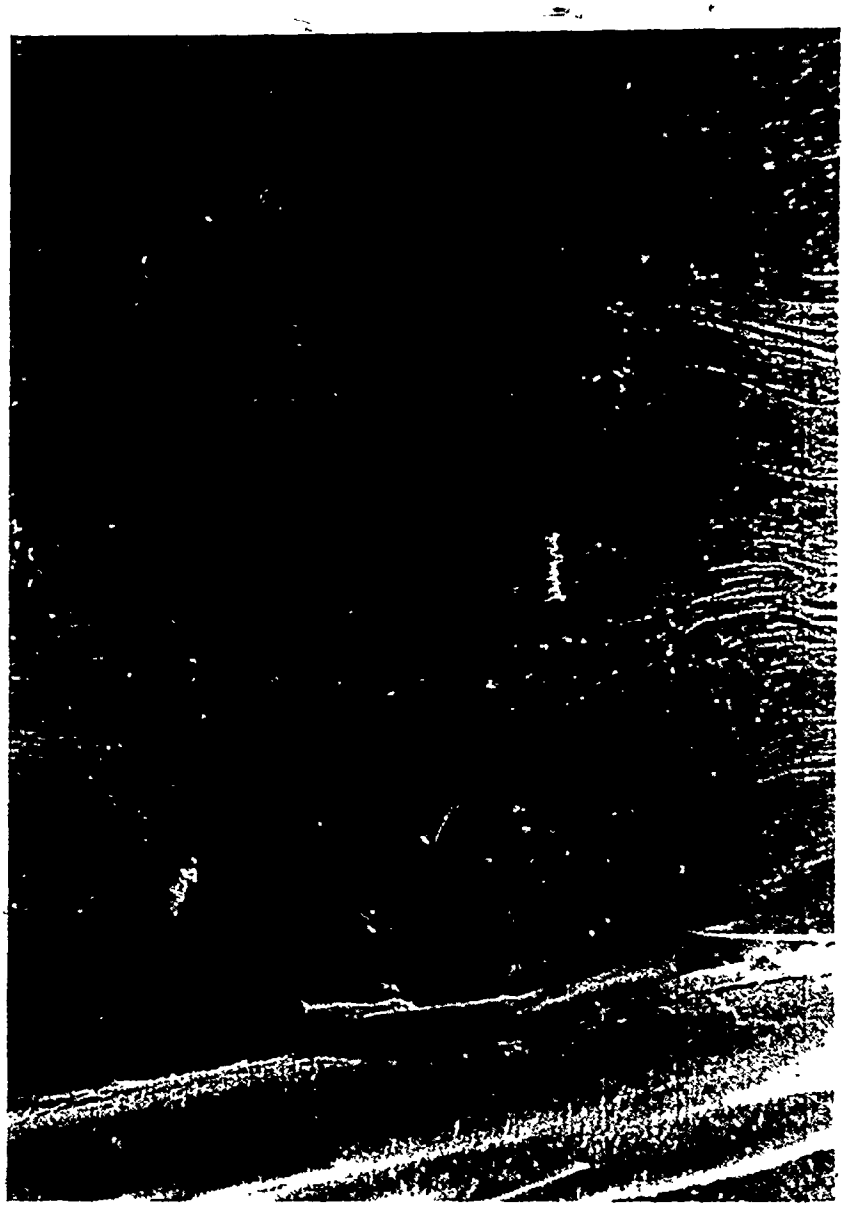
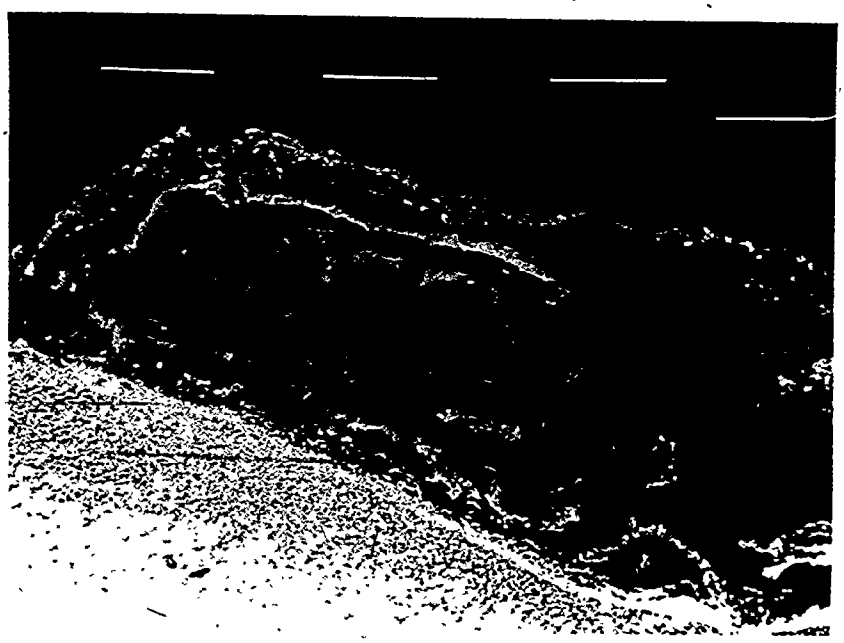


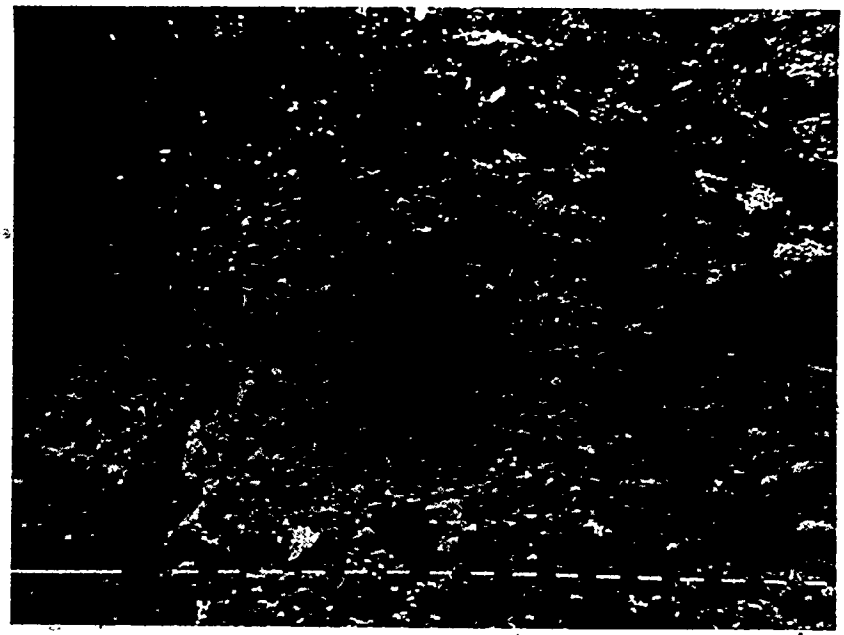
Plate 2B Scanning electron micrograph (SEM) photographs of rat carotid artery after electrical injury.

The top photograph is a view of a completely thrombosed right carotid artery fixed 18 min after a 1mA for 1 min injury. Thrombus consists of both cellular and noncellular material. White dash = 100u.

The bottom photograph shows platelet adhesion to the site of injury fixed 1 min after injury (1mA for 1 min). White dash = 10u.

Courtesy of Dr. R.B. Philp.





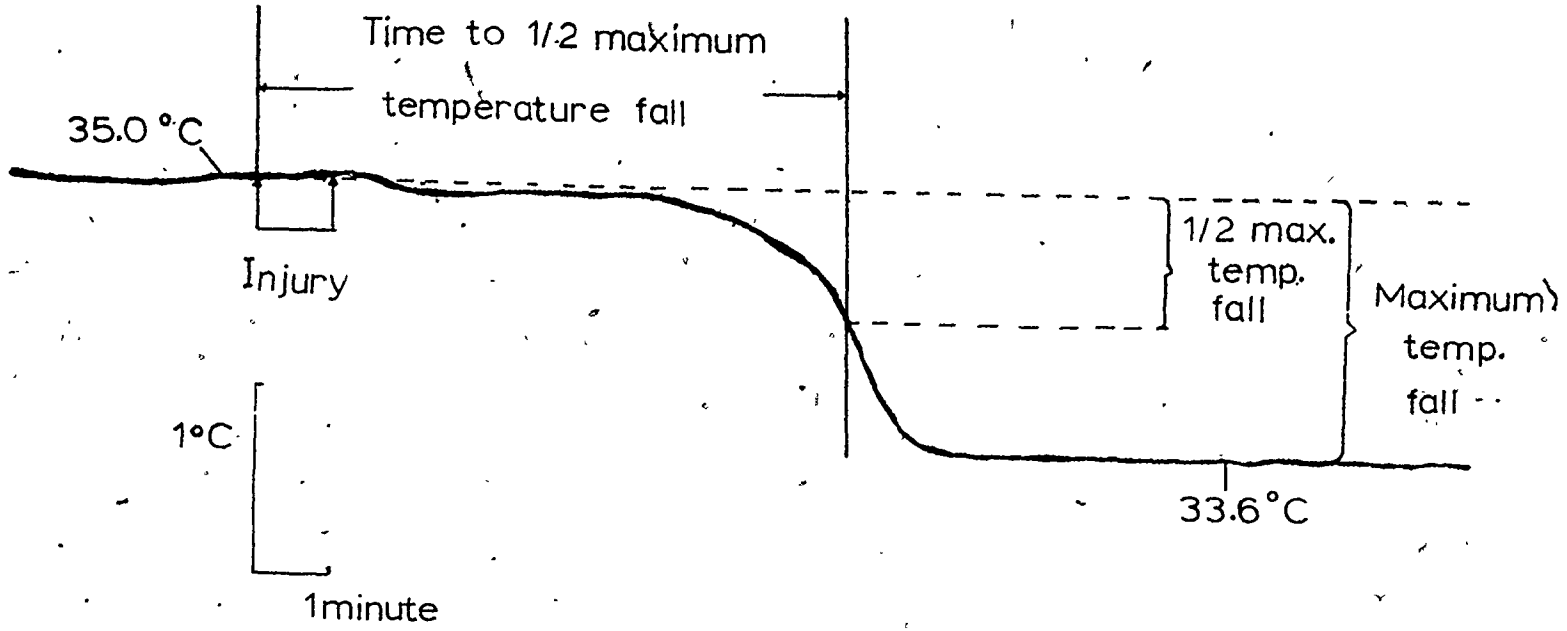
temperature as recorded by a thermistor, in contact with the vessel. The thermistor, attached to a Yellow Springs Tele-Thermometer, was positioned on the blood vessel, cranially from the electrodes. The thermometer was connected to a Rikadenki Model B-141 3-Channel Recorder calibrated so that 1 inch corresponded to a temperature change of 1^{o} C. The temperature was confirmed at several critical points during each experiment by noting the temperature reading on the gauge of the Tele-Thermometer. The obstruction of arterial flow was accompanied by a proportionate reduction in temperature of the vessel. The average temperature fall was $1-2^{o}$ C in magnitude. An arbitrary observation period of 20 minutes after the end of electrical injury was chosen since previous studies showed that the major changes occurred within this time span.

Once the animal was set up with the electrodes and thermometer, the preparation was left for 10-20 min so that the temperature could stabilize and a baseline be established. The recorded temperature was in the range of $34-36^{o}$ C. After injury to the control artery, drugs were injected as described in the ex vivo studies in volumes less than 0.6ml. The second artery was injured 15 minutes after completion of drug infusion unless otherwise noted. The time from the start of electrical injury to the time of a 50% decrease in maximum temperature was designated as the time to thrombosis (Fig. 25). The maximum post-injury fall in temperature was also recorded (Appendix I).

The fall in temperature and the time to 50% maximum fall in temperature were compared in control and drug situations by a student's t-test for paired data. The level of significance accepted was $p < 0.05$.

Fig. 25 Typical blood vessel temperature recording in the rat thrombosis model.

The tracing begins on the left showing the time of injury, the drop in temperature (indicating a thrombotic event) and the measurements made for a control vessel. The temperature was measured using a thermistor connected to a tele-thermometer and recorder.


Time to 1/2 maximum
temperature fall
35.0 °C
Injury
1/2 max.
temp.
fall
Maximum
temp.
fall
1°C
1minute
33.6 °C

2.5.1.3 **Results**

Preliminary work was aimed at studying the aggregation of rat platelets and PGI_2 activity by rat aorta and ultimately determining if a single specific dose of ASA could be found which would inhibit platelet aggregation without inhibiting PGI_2-activity in the vessel wall. In initial studies an attempt was made to establish an assay for AA-induced rat platelet aggregation under the assumption that this aggregation would be most sensitive to the effects of ASA. Rat PRP was diluted 1:1 with saline as this provided more PRP without adversely affecting the aggregation response to ADP. AA in concentrations up to 2.5mM could not induce platelet aggregation in citrated rat PRP (Fig. 26A). High concentrations of ADP (12uM) caused monophasic aggregation which was only partially inhibited by 0.3mM ASA (Fig. 26B).

When PRP was prepared from heparinized blood, concentrations as low as 0.25mM AA caused complete aggregation (Fig. 26C). Increasing the concentration up to 1mM caused a decrease in the "lag phase" (time from addition of AA to the onset of aggregation marked by an increase in light transmission above the baseline). Concentrations of ADP as low as 1.3uM caused full monophasic aggregation, although this aggregation was reversible. In other studies adrenaline did not aggregate rat platelets although it did potentiate the effects of collagen on rat platelets. The effect of ASA on AA-induced platelet aggregation are shown in Fig. 27. Exogenous ASA at 7.5×10^{-5}M completely inhibited AA-induced platelet aggregation. The effect on the 5 minute total height of the AA aggregation curve was all or none in that 6.8×10^{-5}M ASA did not reduce the 5 minute height (Fig. 27A). A dose-response effect was evident in the lag time between addition of AA and the onset of aggregation (Fig.

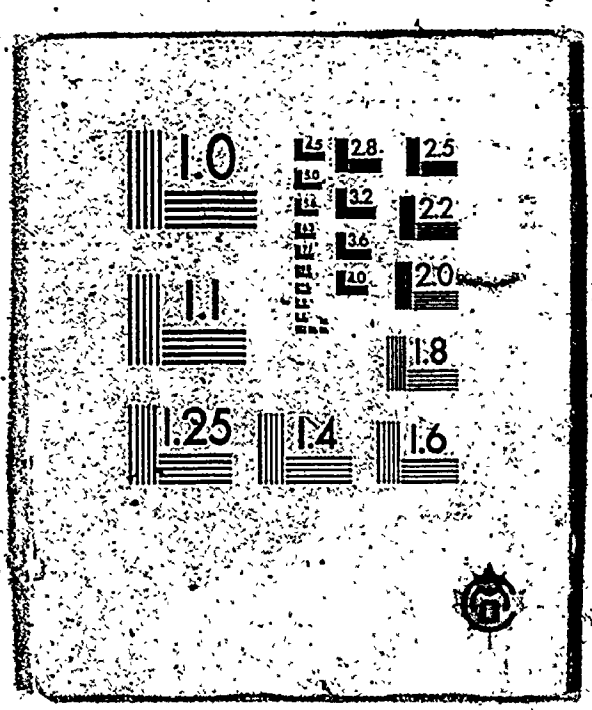
Fig. 26 Typical platelet aggregation curves using rat platelet rich plasma (PRP).

A. The effect of AA on citrated rat PRP diluted 1:1 with saline.

B. The aggregation of citrated rat platelets with ADP and the effects of preincubation with ASA. PRP was preincubated with ASA for 4 min before the addition of ADP.

C. The effect of AA on heparinized rat PRP diluted 1:1 with saline.

D. The aggregation of heparinized rat platelets with ADP and the effects of ASA ex vivo. Control and ASA-treated PRP were obtained from different animals.


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6

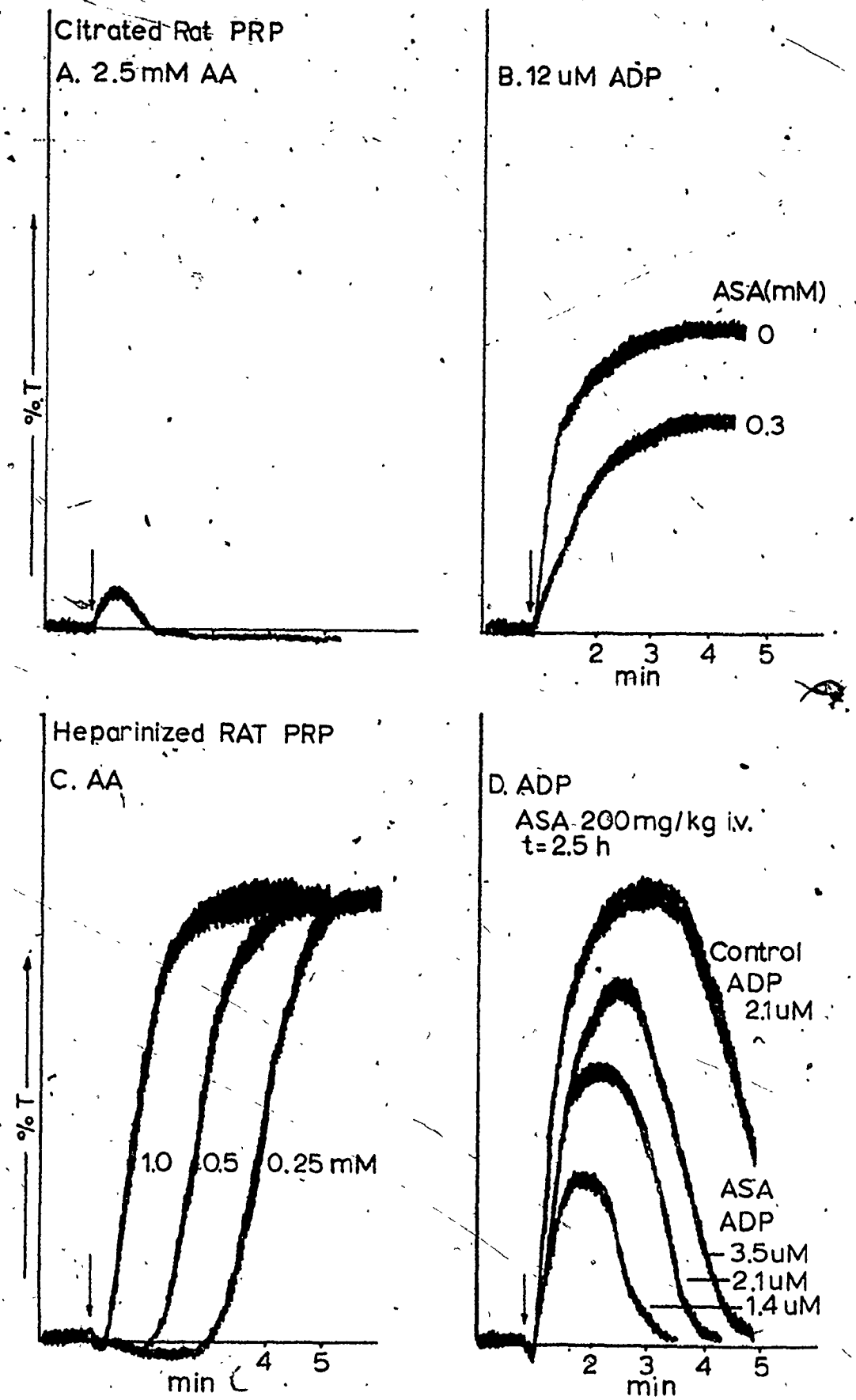

Citrated Rat PRP
A. 2.5 mM AA
% T
B. 12 uM ADP
ASA(mM)
0
0.3
2 3 4 5
min
Heparinized RAT PRP
C. AA
% T
1.0
0.5
0.25 mM
4 5
min
D. ADP
ASA 200 mg/kg i.v.
t = 2.5 h
Control
ADP
2.1 uM
ASA
ADP
3.5 uM
2.1 uM
1.4 uM
2 3 4 5
min

Fig. 27 Studies of rat platelets and rat aorta PGI_2-like activity.

A. Dose-response effect of ASA (4 min pre-incubation) on the 5 min total height of heparinized rat platelet AA-induced aggregation.

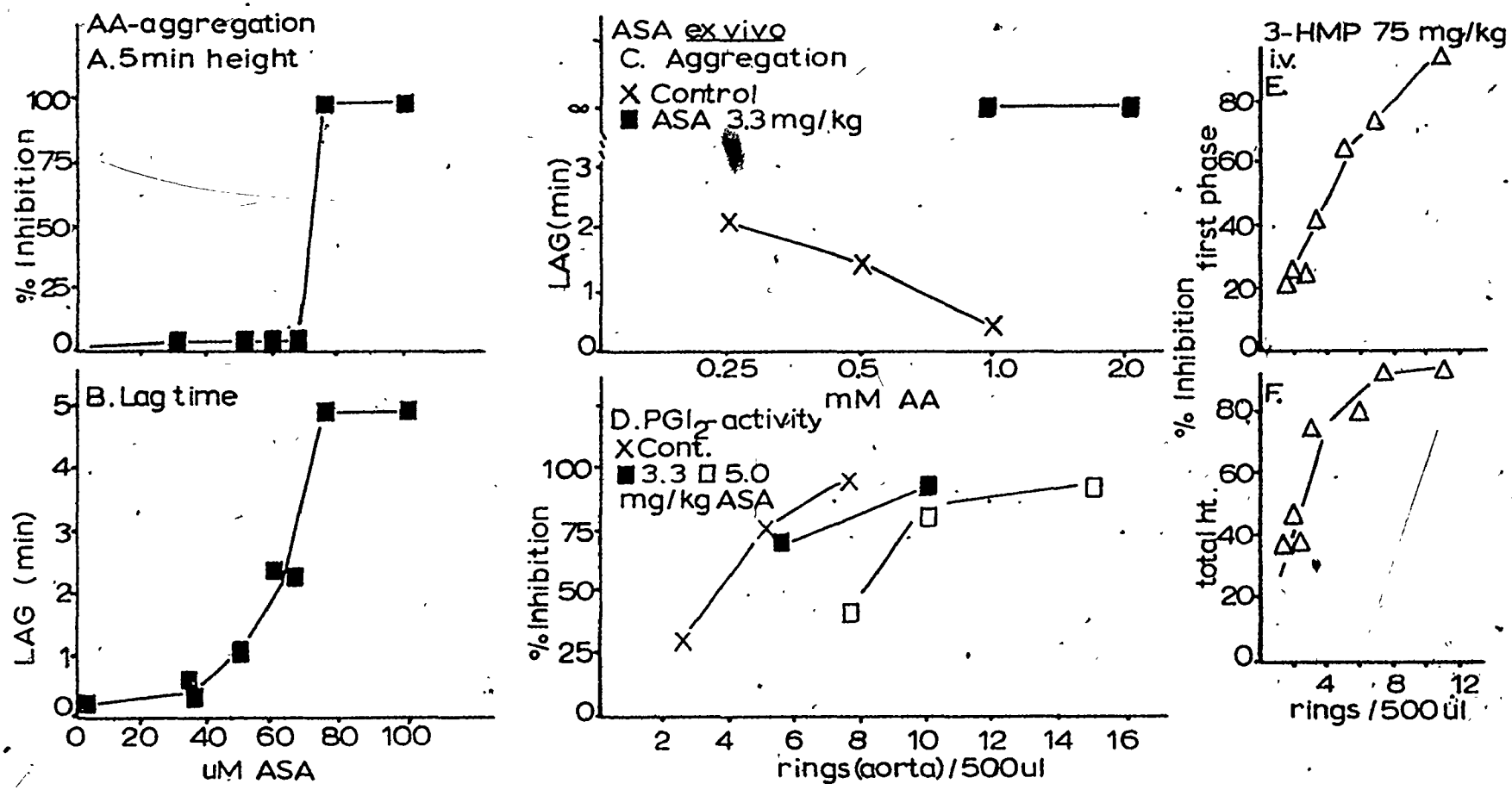
B. Dose-response effect of ASA (4 min pre-incubation) on "lag time" of heparinized rat platelet AA-induced aggregation. Lag time represents the time from the addition of AA to the onset of aggregation marked by an increase in light transmission above the baseline on platelet aggregation curves.

C. The effect of ASA (3.3 mg/kg I.V.) on the lag time of heparinized rat platelet AA-induced aggregation. AA aggregation was totally inhibited.

D. The effect of intravenous ASA (3.3 mg/kg and 5 mg/kg) on PGI_2-like activity production by rat aorta rings measured using a rat platelet ADP-induced aggregation inhibition bioassay.
Dose-response effects were obtained by varying both the 15 mg ring number and the ring incubation buffer volume and converting these values to rings/500 ul. All values were compared to control platelet aggregation curves in which buffer alone was incubated.

E& F. Human platelet aggregation bioassay of PGI_2-like activity from rat aorta rings from an animal after 3HMP, 75 mg/kg I.V.
E. % inhibition of first phase height
F. % inhibition of 5 min total height of platelet aggregation curves.

Each point represents a single value.


AA-aggregation
A. 5 min height
% Inhibition
100
75
50
25
0
B. Lag time
LAG (min)
5
4
3
2
1
0
0 20 40 60 80 100
uM ASA
ASA ex vivo
C. Aggregation
X Control
■ ASA 3.3 mg/kg
LAG (min)
3
2
1
0
0.25 0.5 1.0 2.0
mM AA
D. PGI2 activity
X Cont.
■ 3.3 □ 5.0
mg/kg ASA
% Inhibition
100
75
50
25
0
2 4 6 8 10 12 14 16
rings (aorta) / 500 ul
3-HMP 75 mg/kg
i.v.
E.
% Inhibition first phase
80
60
40
20
0
F.
total ht.
80
60
40
20
0
4 8 12
rings / 500 ul

27B). Concentrations of ASA as low as 3.5×10^{-5}M caused an increase in this lag. The ex vivo effect of ASA (I.V.) on AA aggregation is demonstrated in Fig. 27C. ASA at 3.3mg/kg completely abolished AA-induced rat platelet aggregation. Aorta rings from these animals produced platelet inhibitory activity when incubated under the usual conditions, however the activity from rings from animals given 5mg/kg ASA I.V. was reduced (Fig. 27D). Based on these studies ASA at 3.3mg/kg was used initially in the rat thrombosis model. In other selected studies under similar conditions, 3HMP 10mg/kg and 3-PBA 10mg/kg had no effect on platelet function. 2-PBA 10mg/kg, like ASA, abolished AA-induced platelet aggregation but at 10mg/kg 2-PBA did not inhibit rat PGI_2-like activity from aorta rings.

Previous work in the laboratory demonstrated that 200mg/kg ASA prolonged the time to thrombosis in the rat thrombosis model (Philp et al 1978). Heparinized PRP from a rat that had been given ASA 200mg/kg I.V. aggregated to low concentrations of ADP (Fig. 26D) indicating that there is still some degree of platelet function at this dose of ASA. The response did appear partially inhibited compared to controls and deaggregation appeared more rapid.

An attempt was made to study the effects of a dose of 100mg/kg 3HMP I.V. and to assay effects on platelet aggregation and PGI_2-like activity. After infusion of approximately 75% of the dose, respiration was arrested and the rat died. The injected vessel became thrombosed. The lungs contained small areas of hemorrhage and upon histological examination, it was found that a massive outpouring of plasma proteins into lung tissue had occurred. As this agent appeared most potent as an inhibitor of PGI_2 in in vitro studies, the aorta was removed and assayed

for platelet inhibitory activity on human PRP (Fig. 27E,F). The aorta still produced platelet inhibitory activity.

Control experiments using the rat thrombosis model were designed to evaluate differences in the time to 50% thrombosis and the degree drop in temperature between the right carotid artery or pre drug control and the left carotid artery, 15 minutes after infusion of 50% PEG in saline - the vehicle for all drugs in this study. Vehicle was injected in a volume of 1ml/kg as this was the volume used in all studies. There were no statistical differences in the time to 50% thrombosis or the fall in temperature between these 2 sides using a paired t-test (Table I). ASA was tested at 3.3mg/kg based on preliminary data which indicated that this dose blocked AA-induced platelet aggregation and had minimal effects on aorta PGI_2 production. This dose had little effect on temperature drop or time to thrombus formation. ASA 10mg/kg also had no effect and at 100mg/kg ASA actually caused a decrease in the time to thrombus formation, although this did not reach statistical significance. ASA 200mg/kg, did inhibit the time to thrombus formation ($p< 0.05$). 2-PBA 50mg/kg, had no effect on either parameter although 100mg/kg consistently prolonged the time to thrombosis ($p< 0.02$). 3-PBA 100mg/kg, demonstrated a trend towards an increase in time to thrombosis but this did not reach statistical significance. ABA had no significant effect at 50mg/kg but increased the time to thrombosis at 100mg/kg ($p< 0.01$) and 200mg/kg ($p< 0.05$). 3-MP 100mg/kg, did not demonstrate a statistically significant effect although a trend was apparent while 3HMP at 10 or 25mg/kg had no significant effect. This drug was not well tolerated by the animals at doses higher than 25mg/kg. When ASA 10mg/kg was tested at 10 minutes after drug infusion, the time to thrombosis was

TABLE 1 EFFECTS OF BENZOIC ACID ANALOGS ON THE RAT THROMBOSIS MODEL (15 min post drug injury)

I. TIME TO THROMBOSIS

Time to 1/2 Max. Temp. Drop of Carotid Artery After Injury (mean ± SEM)

Drug	n	Control (min) Right Artery	Drug (min) Left Artery	
Control 50% PEG/Sal.	6	11.3 ± 0.5	10.5 ± 1.0	
ASA 3.3mg/kg	4	8.6 ± 1.9	9.0 ± 1.9	
ASA 10mg/kg	6	9.2 ± 1.3	9.4 ± 2.1	
ASA 100mg/kg	6	8.4 ± 1.0	7.5 ± 1.7	
ASA 200mg/kg	6	9.3 ± 1.4	15.8 ± 2.8	p<.05*
2-PBA 50mg/kg	6	9.2 ± 1.0	9.4 ± 0.4	
2-PBA 100mg/kg	6	9.8 ± 1.0	13.6 ± 1.2	p<.02
3-PBA 100mg/kg	6	9.8 ± 0.6	13.5 ± 1.7	p<.1
ABA 50mg/kg	6	8.1 ± 0.1	10.7 ± 1.2	
ABA 100mg/kg	6	8.5 ± 0.7	12.4 ± 1.1	p<.01
ABA 200mg/kg	7	9.2 ± 0.6	11.5 ± 0.9	p<.05
3-MP 100mg/kg	5	7.2 ± 0.6	10.6 ± 1.4	
3HMP 10mg/kg	4	9.0 ± 0.9	9.5 ± 0.8	
3HMP 25mg/kg	4	10.0 ± 0.2	10.5 ± 1.1	

* compared to controls by student's t-test for paired data

prolonged ($p < 0.005$) (Table 2). Effects on the degree of temperature drop did not reach statistical significance under most conditions (Appendix I).

In order to represent this data graphically, the "% increase in time to thrombosis" was calculated for each drug concentration (Fig. 28) and these points were plotted and compared to control animals which had been tested with vehicle alone (Fig. 28). According to the degree of effect on thrombosis, determined by assigning arbitrary values to each position on the graph, the benzoic acid analogs were ranked in order of potency as antithrombotics. Despite a lack of complete consistency, ASA 200mg/kg was considered the most potent agent as it was the only one to completely eliminate the fall in temperature and did so 3 out of 6 times. Other agents were ranked on the basis of consistency in the "% increase in time to thrombosis" being larger than controls. Previous work in our laboratory demonstrated that ASA at 10mg/kg was antithrombotic if tested 10 minutes after drug administration (Philp unpublished). We confirmed this result.

ASA at 100mg/kg was also the only agent to induce an increase in thrombotic tendency. All other agents were somewhere in between these 2 effects of ASA. All of the ASA-like agents, excluding 3-MP, caused some degree of hemolysis at concentrations above 100mg/kg. 3-MP at 100mg/kg was approaching the LD_{50} dose (the dose causing death in 50% of the animals) for this agent in rats.

TABLE 2 EFFECTS OF ASA ON THE RAT THROMBOSIS MODEL (10 min post drug injury) I. TIME TO THROMBOSIS

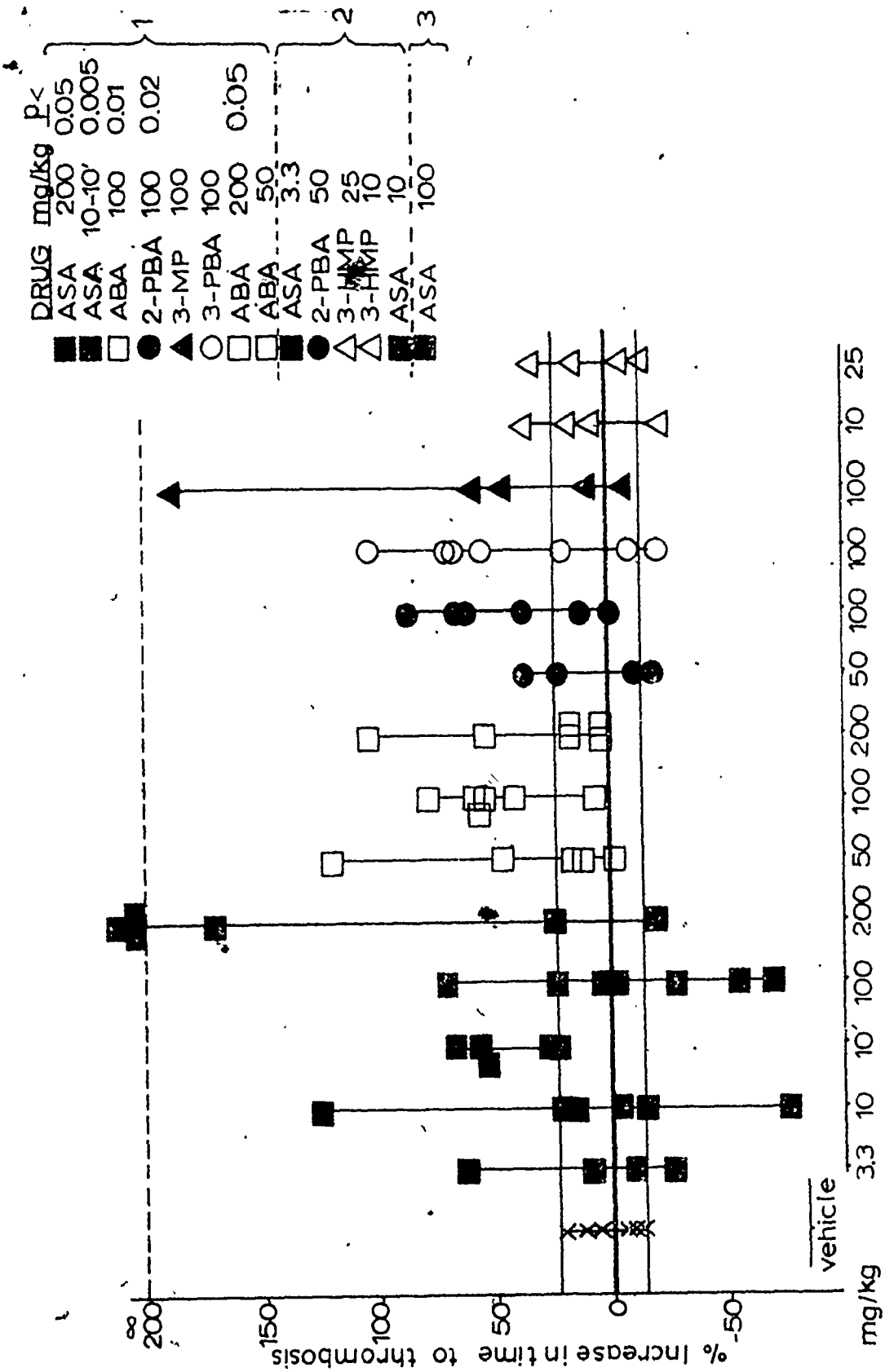
Time to 1/2 Max. Temp. Drop of Carotid Artery After Injury (mean ± SEM)

Drug	n	Control (min) Right Artery	Drug (min) Left Artery
ASA 10mg/kg	6	7.6 ± 0.8	10.9 ± 0.6 $p<.005$*

*compared to control by student's t-test for paired data

Fig. 28 The effects of the benzoic acid analogs on the rat thrombosis model.

In this graphical representation of the data from Tables 1 and 2, the "% increase in time to thrombosis" represents the difference in time to thrombosis of the post drug left carotid artery compared to the right artery control, as a % of the control.
Each point represents a different animal. Each symbol represents a different drug. The effects of a specific drug at a specific dose, as marked on the abscissa, are lined up vertically to facilitate comparison with the controls on the left of the graph. The range of the controls has been marked.
Drugs have been ranked, by visual comparison with controls, as having: 1. antithrombotic effects 2. no effects, or 3. prothrombotic effects.
Thrombosis was induced in the left carotid artery in all animals 15 min after drug administration except for ASA 10' in which thrombosis was induced 10 min following the administration of 10 mg/kg ASA.
For values, see Tables 1 and 2.


DRUG mg/kg p<
ASA 200 0.05
ASA 10-10' 0.005
ABA 100 0.01
2-PBA 100 0.02
3-MP 100
3-PBA 100 0.05
ABA 200
ABA 50
ASA 3.3
2-PBA 50
3-HMP 25
3-HMP 10
ASA 10
ASA 100
1
2
3
% Increase in time to thrombosis
∞ 200 150 100 50 0 -50
vehicle
3.3 10 10' 100 200 50 100 200 50 100 100 100 10 25
mg/kg

2.5.1.4 **Discussion**

Due to the small numbers of animals in each group and the variation in results between animals, drug effects were analyzed by the student's t-test for paired data using the left carotid artery as the post drug result and the right carotid as the drug control. For each animal the "% increase in time to thrombosis" (IT) between left and right side was calculated and plotted and a visual comparison of effects between treatments was also made.

IT values from control animals receiving vehicle only were plotted and the range marked (Fig. 28). There was only a small amount of variation among these values compared to drug treatment groups. ASA at 3.3 mg/kg had little effect on this distribution. At this dose, ASA blocked AA-induced aggregation in heparinized rat PRP, ex vivo, but allowed almost full production of PGI_2 by rat aorta rings ex vivo. This dose appeared to have a specific effect on platelet cyclo-oxygenase without affecting thrombus formation. Based on this data and on the reported sensitivity of the enzyme in arterial tissue, a dose of ASA of 10 mg/kg, would be expected to cause greater inhibition of PGI_2 synthesis. ASA, 10 mg/kg, did not influence the outcome of the 15 min post drug injury and thrombosis as the majority of IT values fell within the range. The points that fell outside this range did not consistently fall on the thrombotic or antithrombotic side.

Other drugs and doses which did not appear to influence the thrombotic event were 3HMP at 10 and 25 mg/kg and 2-PBA at 50 mg/kg. Although 3HMP was the most potent inhibitor of PG synthesis in platelets (2.3.2.3) and PGI_2-activity from the vessel wall (2.4.1.3), rat

platelets aggregated to AA after an intravenous dose of 10 mg/kg 3HMP to the rat, and rat aorta produced PGI_2-like activity after an intravenous dose of 75 mg/kg. Gryglewski et al (1976) reported that plasma enzymes such as glutathione peroxidase inhibit hydroperoxide activity and this may account for this lack of effect. On the other hand, 3HMP has in vivo pharmacological activity in other systems (3.4.1.3). 2-PBA inhibits platelet PG synthesis and, possibly, vessel PGI_2 synthesis at this concentration.

ASA at 100 mg/kg had a tendency to promote thrombus formation and this tendency has been reported before under similar conditions (Philp et al 1978). No other agent demonstrated this same effect. At 100 mg/kg ASA, full inhibition of PG synthesis in platelets and the vessel wall is likely, based on the demonstrated effects of lower doses.

All other agents at various concentrations demonstrated some tendency to inhibit thrombosis as reflected in an increase in IT values. With the exception of ASA 10 mg/kg, which displayed antithrombotic activity when thrombosis was induced 10 minutes after drug administration, all concentrations necessary to achieve such an effect. bordered on toxic levels. From the information available concerning the effects of these agents on platelet and vessel wall PG activities, it seems unlikely that the inhibition of PG synthesis alone can account for these effects. For example, 3-MP and ABA were only weak inhibitors of PG synthesis (2.3.2.3) and yet they did inhibit thrombosis to some extent while ASA 3.3 mg/kg and 2-PBA 50 mg/kg inhibited platelet PG synthesis ex vivo and yet were not antithrombotic. On the other hand, ASA was the most potent inhibitor of PG synthesis (2.3.2.3) and was the only agent to completely block thrombus formation in this model (at 200 mg/kg).

2-PBA inhibited thrombus formation at some concentrations and was second only to ASA as a PG synthesis inhibitor. 3-PBA caused a consistent increase in PGI_2-like activity at lower concentrations in in vitro studies on rabbit aorta (2.4.1.3) and in this model had a tendency to prolong the time to thrombosis although it was not statistically significant. 3-MP inhibited platelet PDE and it may inhibit platelet function through this mechanism at the high dose used. 3-MP has structural similarity to the flavenoid class of compounds. These compounds have many activities including inhibition of lipid peroxidation and inhibition of endothelial damage as well as PDE inhibition.

ABA is of particular interest. ABA did not inhibit platelet or vessel wall PG synthesis to any great extent although Cerskus (1978) reported that ABA did inhibit both phases of platelet aggregation at high doses. 3-MP was designed to resemble the ring tautomer form of ABA and, when it was found to inhibit PDE, elevation of cAMP by PDE inhibition was proposed as a possible mechanism of action. ABA however, did not inhibit PDE over the dose range tested and, because the antithrombotic effect was not dose dependent (the antithrombotic effect was greater at 100 mg/kg than 200 mg/kg), it is unlikely that this is the only mechanism of action. ABA was also reported to inhibit inflammation in the carrageenin rat paw model (Cerskus 1978, Cerskus and Philp 1981). This inflammation model is sensitive to PG synthesis inhibitors and this led these workers to conclude that ABA represents a separation of antiPG and anti-inflammatory activity. This information, together with the effect of ASA 10 mg/kg, 10 minutes prior to injury suggests some other mechanism of antithrombotic action of salicylates.

The fact that ASA was also a potent antithrombotic agent only at very high doses using the 15 minute incubation period may also be relevant. Various reports on the effect of salicylate on ASA inhibition of platelets and PGs (Merino et al 1980, Philp and Francey 1981) point to an interaction of salicylate with the PG synthetic enzymes which is not detectable in the standard PG inhibition studies. Lands (1979) suggested that differences in substrate or activator content of assays may lead to paradoxical results such as salicylate inhibition of inflammation without PG synthesis inhibition. Walter et al (1980) have recently reported a biphasic platelet inhibitory effect of ASA given orally to volunteers. The effect was detected on the rate of platelet aggregation which was maximally inhibited 12-24 minutes after ingestion of drug and, although plasma salicylate levels continued to increase after this time, the inhibition of platelet function decreased. This suggested some early reversible effect of ASA. In the comparison of drug effects two other factors become apparent. First of all, only ASA and ABA were tolerated at 200 mg/kg. As mentioned before, while this dose was associated with increased antithrombotic activity for ASA, ABA appeared to cause less consistent effects at this dose compared to 100 mg/kg. This suggests that a specific structure is required for antithrombotic activity and that it is not the result of a nonspecific effect of any benzoic acid derivative. Secondly, only ASA demonstrated a tendency to decrease the time to thrombosis. All other agents tested at 100 mg/kg showed some antithrombotic activity. These factors must be considered in relation to ASA as the most potent irreversible inhibitor of platelet and vessel wall PG synthesis and possibly some other activity unique to ASA.

Salicylates have other actions (see Chapter 1). These include the

stabilization of endothelial membranes and the interaction with histamine release and with cellular proteins. Some or all of these actions may be applicable to the antithrombotic results seen here. The activity of high doses of salicylates to increase fibrinolysis in blood (Menon 1970, Gupta and Gupta 1977, Moroz 1977) appears particularly important when considered along with the effects of heparin (Hladovec 1971, Philp et al 1978), which completely inhibited thrombus formation when used at high doses in this model. The indirect effects of ASA on the production of 12-HPETE and the inhibitory effects of ASA on the conversion of 12-HPETE to 12-HETE (Siegel et al 1979b) may be important factors in offsetting the antiplatelet PG synthesis effects of ASA.

The importance of PGs in the hemostatic process is evident from such facts as the increase in bleeding time after ASA ingestion and the bleeding tendency in people with cyclo-oxygenase deficiencies. The report by Kinlough-Rathbone et al (1980) that high doses of collagen can overcome ASA inhibition of platelet aggregation and other similar reports (see 2.3.1.1) suggest that in a model such as this one involving a strong, acute injury to the vessel where other factors including thrombin, red cell lysis and a large collagen component are important, ASA inhibition of platelets may be insufficient to prevent thrombosis (Packham and Mustard 1980). However, ASA has been shown to be of benefit to patients with certain types of thrombotic disease. Screening of potential antithrombotic agents may be more accurately performed using a series of models involving

(i) strong injury such as this model

(ii) more sensitive models such as that reported by Bourgain and Six (1974), which uses a combination of electrical injury and a platelet

aggregating agent to induce small vessel white thrombus formation and possibly

(iii) a model in which atherosclerotic vessels are involved.

In conclusion, this study demonstrates that:

1. Inhibition of platelet cyclo-oxygenase alone is insufficient to explain antithrombotic action in this model.

2. ASA has unique features in comparison with the other agents and this includes a lack of overt toxicity at very high doses (shared by ABA), complete inhibition of thrombosis at high doses and a tendency to decrease the time to thrombosis at 100 mg/kg.

3. 2-PBA, which was the only other irreversible platelet PG synthesis inhibitor, increased the time to thrombosis consistent with the effects of ASA.

4. ABA at 100 mg/kg consistently increased the time to thrombosis and this may be related to its anti-inflammatory activity.

5. Because of the tendency of a wide variety of these benzoic acid analogs to increase the time to thrombosis under a variety of conditions, different mechanisms of action likely mediate the effect in each situation. These data include ASA 10 mg/kg with 10 minutes post drug injury, 3-MP 100 mg/kg, ASA 200 mg/kg, 3-PBA 100 mg/kg and ABA 100 mg/kg. Inhibition of PG synthesis is a possible mechanism of action in some situations.

6. More sensitive models of thrombosis may be useful in conjunction with this model in determining the agent most useful for a given thrombotic disease.

Chapter 3 INFLAMMATION AND ASA

3.1 Polymorphonuclear Leukocytes, PGs and Inflammation: Historical Notes and Current Concepts

Life on earth could not have been sustained to this day, 3 billion years from its origin, without the ability of living organisms to overcome injury and repair themselves. With the development of multicellular organisms, it became no longer necessary for individual cells to be able to deal with bacteria and other noxious agents as certain cells differentiated into "specialists" with the purpose of protecting the entire organism. In mammals, in which the internal environment is far more specialized than in invertebrates, for example, local injury brings about a response of great complexity due, in part, to the presence of blood vessels.

Cornelius Celsus, in the first century AD, defined the cardinal signs of inflammation: redness, swelling, heat and pain. John Hunter (1793), maintained that inflammation was not a disease but a beneficial reaction to injury and Julius Cohnheim wrote the first fundamental work on the subject in 1867, based on his studies of live frog mesentery and tongue as viewed under the microscope. Simple exposure of this tissue to air led to changes in the small blood vessels which included dilatation, increased blood flow, adhesion of white cells to the vessel wall, movement of these cells through the walls and out of the blood vessels and finally, movement of fluid out of the vessels. He later realized that plasma escaped from the vessels because vessel permeability

leukocytes. These cells originate in the blood and, by poorly understood mechanisms, stick to blood vessels, pass through and crawl outwards into the tissues towards the clump of bacteria or damaged cells. These cells then attempt to consume and digest the offending agent or tissue debris in a process termed phagocytosis. The cells of the inflammatory response include polymorphonuclear neutrophils (PMN), basophils, eosinophils as well as monocytes, lymphocytes and plasma cells. PMNs arise from the bone marrow from precursor cells through a series of divisions and differentiations and this appears to be modulated by stimulatory or inhibitory factors such as colony stimulating factor (CSF). Neutrophils have a half life of about 7 hours as fully functional PMNs. All of the characteristic neutrophil activities have roles in the inflammatory response (Murphy 1976).

Leukocytes leave the vessels via interendothelial junctions as first recorded by Arnold in 1875 and confirmed in electron microscopy studies by Marchesi and Florey (1960). Although PMNs do not cause the leaks in the endothelium, they cause temporary damage to the basement membrane (Hurley 1964). In the early stages of the acute inflammatory response, the predominant cell infiltrating the tissue is the PMN and this is followed by other cells such as the blood monocyte (or tissue macrophage) 6 - 24 hours after the onset of inflammation. The peak of PMN emigration depends on the presence of pyogenic (or pus producing) bacteria. Although this biphasic cellular response to injury may reflect differences in cell mobility, increasing evidence points to the synergistic action of specific chemical mediators for each cell type. Chemotactic movement or movement initiated and directed by a chemical substance was first observed by Leber in 1888 when he injected molds or

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putrefied rabbit muscle into guinea pig corneas. More controlled experiments were performed by Harris (1953) who recorded the movement of PMNs on microscope slide coverslips by photography. Boyden (1962) used a two chamber system of liquids separated by a filter paper and found that leukocytes would crawl through the filter paper from the original chamber to the other, if a chemotactic substance was placed in the second chamber. Ryan and Hurley (1966) found that tissue factors and serum factors interacted to form chemotactic substances and Bessis (1964) reported that dying, but not dead PMNs, elicited a chemotactic response. Chemotaxis is both an in vitro and in vivo phenomenon (Buckley 1963).

The report of the ability of leukocytes to phagocytose bacteria as suggested by Metchnicoff, did much to contribute to the appreciation of their role in the inflammatory process however, Wright and Douglas (1904) added to this picture when they demonstrated the importance of the presence of serum factors in phagocytosis. These factors were found to coat the bacteria and were found necessary for PMN phagocytosis of many kinds of bacteria. These factors were called "opsonins" and included a) heat stable factors such as IgG_1 and IgG_3, which are specific antibodies directed against the surface components of the bacteria and b) fragments of the third (C3) and fifth (C5) component of complement, which are heat labile (see below).

PMN structure and function have been thoroughly reviewed by Klebanoff and Clark (1978). Neutrophils possess large numbers of cytoplasmic granules of two main types a) azurophilic granules which are large, dense granules that contain lysosomal hydrolases, cationic proteins, peroxidase and lysozyme and b) specific granules which are

smaller, less dense and contain alkaline phosphatase, lysozyme and lactoferrin. Large amounts of glycogen in the cytoplasm allow energy production by these cells in damaged tissue where the oxygen supply is low. After attachment of a particle to the PMN, the particle is internalized into a membrane compartment or phagosome. The granules then fuse with the phagosome and release their contents inside of it. Bacterial cell death is mediated by the acid pH inside the phagosome, cationic proteins, lysozyme, lactoferrin, superoxide anion and hydrogen peroxide (H_2O_2). H_2O_2, in the presence of myeloperoxidase and halide ions, provide the most important component of the neutrophil microbicidal activity (Klebanoff 1975).

Mediators of the inflammatory process can be divided into plasma and tissue derived factors. The plasma factors include the kinin system (bradykinin), the clotting system (fibrinopeptides and fibrin degradation products) and the complement system. The complement system consists of 9 major components and the activation of each component takes place in a sequence analogous to the blood coagulation sequence. The complement system may be activated by immune complexes and here some components of the system, namely C1, C4 and C2 interact to cleave the component C3 into fragments and to initiate further activation of the system. This is the classical pathway of complement activation. C3 may be directly activated by certain antigen-antibody complexes, various polysaccharides, bacterial lipopolysaccharides and cobra venom and this is the "alternate pathway". As well as playing a role in the opsonization of foreign material, complement plays a role in cell lysis, increasing vascular permeability and chemotaxis of leukocytes.

Tissue factors can be classified as vasoactive amines, acidic lipids, lysosomal components, lymphocyte products and other factors, more difficult to classify, such as endogenous pyrogens, substance P, neurotensin, collagen fragments, cAMP and others. Both histamine and 5HT are contained in mast cells and platelets. These agents contract smooth muscle and cause vasodilatation. Acid lipids include slow reacting substance (SRS) as first reported by Feldberg and Kellaway (1938) and PGs. Lysosomal products are usually derived from PMNs although other cells including platelets may also release these products. Ryan and Majno (1977) have summarized the role of mediators in inflammation: 1. vascular leakage is caused by vasoactive amines especially histamine, bradykinin and/or PGs 2. leukocyte infiltration is caused by the products of the complement system especially C5, PMNs, cationic protein from mononuclear cells and eosinophil chemotactic factor of anaphylaxis (ECFA) from eosinophils 3. tissue damage is due mainly to PMN lysosomal products.

Inflammatory stimuli induce PG synthesis and release and PGs contribute to the genesis of fever, pain, vasodilatation and increased vascular permeability. Nonsteroidal anti-inflammatory drugs (NSAID) inhibit PG synthesis and decrease the signs and symptoms of inflammation. In a number of inflammatory conditions, the concentrations of PGE_2 and $PGF_{2\alpha}$ increase (Flower 1977) and increased levels of TXA_2 and PGI_2 have also been reported (Sturge et al 1978, Higgs and Salmon 1979). Cyclo-oxygenase products may be formed locally during the initial stages of inflammation. It is clear that there are large concentrations of cyclo-oxygenase-derived products in the inflamed joint and reduction

of these concentrations is associated with reduced inflammation (Kuehl and Egan 1980). The role of PGs in the immunological mechanisms of inflammation has been reviewed by Trang (1980). PGs play a role in cell mediated immunity at the level of the macrophage, in antigen or mitogen induced blastoid transformation and in the cytotoxic effect of activated lymphocytes. PGs play a role in regulating the microcirculation and are among the most potent known vasoactive substances.

Part of the confusion involving the role of PGs in inflammation lies in the fact that, in experimental conditions, PGs have been shown to have both inflammatory and anti-inflammatory activity. Kaley and Weiner (1971) and Ferreira and Vane (1974) showed that PGEs caused erythema but not edema or pain and Williams and Peck (1977) confirmed this with the report that, compared to histamine and bradykinin, the erythema caused by PGE_1 was much longer lasting but was not accompanied by edema. PGE_1 however, was found to dramatically increase the edema response of tissues to histamine and bradykinin (Ferreira and Vane 1974) and the same was true of PGI_2 (Davidson et al 1978, Williams 1979). The site of action for increased vessel permeability was the post capillary venules (Crunkhorn and Willis 1971). PGE_2 was found to increase the edema induced by carrageenin in the rat paw and the PG endoperoxides have also been shown to have this activity (Vane 1976). On the other hand, when PGE_2 was administered twice daily in 500ug doses for 3 days, it was found to have a therapeutic effect on adjuvant induced arthritis but not on carrageenin rat paw inflammation or cotton pellet induced granuloma formation (Aspinall and Cammarata 1969). PGE_1 had a similar effect on adjuvant arthritis, independent of adrenal gland function but, in contrast to the work of Aspinall and Cammarata (1969), was also

anti-inflammatory in the carrageenin rat paw model (Zurier and Quagliata 1971, Zurier et al 1973). PGE_1 and PGE_2 were found to exert anti-inflammatory effects by virtue of their ability to decrease the number of PMNs entering the focus of inflammation (Zurier et al 1973). Bonta et al (1977) proposed a dual function of PGs in chronic inflammation based on studies of inflammation in essential fatty acid (EFA) deficient rats. In this model, decreased exudate and PGE levels were associated with the kaolin pouch granuloma but the proliferative component of inflammation was significantly increased.

PGs may exert anti-inflammatory activity by increasing cAMP in PMNs. PMN lysosomal enzyme release is believed to be under bidirectional control by cyclic nucleotides. cAMP has been shown to inhibit and cGMP to increase the release of lysosomal enzymes by PMNs into the media (Zurier et al 1974, Goldstein 1976). PGI_2 has been found more potent than PGE_2 or E_1 in elevating cAMP and inhibiting lysosomal enzyme release (Weissmann et al 1980). Like PGE_1, PGI_2 increases edema, vasodilatation and the response to bradykinin but is anti-inflammatory with respect to the release of mediators of inflammation. Because PMNs have been shown to be essential for the initiation of the inflammatory response (Turner and Lynn 1978) it is important to establish clearly the relationship between PGs and PMNs. Reports of effects of PGs on PMN chemotaxis are conflicting (Trang 1980). Although reports have appeared that PMNs produce PGs, the importance of this finding has not been established (3.3.1).

Concerning PGs and other inflammatory cells, increasing evidence points to a role for platelets in inflammation (Silver et al 1974, Nachman 1978). Thrombosis occurs in the microcirculation in inflamed

areas, platelets release potent inflammatory mediators and anti-inflammatory agents inhibit platelet aggregation.

PMNs and macrophages, in the course of phagocytosing bacteria and other foreign material consume large amounts of molecular oxygen. This is transformed by membrane bound NADPH oxidase to superoxide anion ($O_2^{\cdot -}$) and subsequently to other oxidizing species (Kuehl and Egan 1980). In response to inflammatory stimuli, these cells release a substantial portion of the $O_2^{\cdot -}$ to the external medium, however, superoxide dismutase (SOD), an enzyme which dismutes $O_2^{\cdot -}$ to H_2O_2 and O_2 is present in large quantities in the cell cytoplasm, is protective here and is anti-inflammatory in some models of inflammation (Kuehl and Egan 1980). The low oxidizing potential of $O_2^{\cdot -}$ coupled with its capacity to form hydroxyl radicals (·OH) via the iron catalyzed Haber-Weiss reaction, led to the suggestion that the ·OH, which reacts with any organic compound, is the actual inflammatory species (McCord and Day 1978). Both SOD and catalase prevent ·OH formation by removing the essential components of the Haber-Weiss reaction, $O_2^{\cdot -}$ and H_2O_2 respectively. However SOD, which inhibits inflammation by inhibiting leukocyte infiltration, has anti-inflammatory activity while catalase does not (McCord et al 1979) suggesting that $O_2^{\cdot -}$ is in fact the important species. Petrone et al (1980) demonstrated that $O_2^{\cdot -}$ reacted with serum fatty acids to produce an inflammatory substance.

Kuehl et al (1977) have suggested that the oxidant, produced in the peroxidase step in PG synthesis, has potent destructive effects on organic compounds beside the PG synthetic enzymes and these results warrant further investigation (see Lewis and Del Maestro 1980).

All prior discussions of NSAID and PGs (Chapter 2) are relevant to

their effects on inflammation and will not be discussed in detail here. NSAID accumulate in the stomach, kidney and inflamed joint (Graf et al 1975) and an understanding of the pharmacokinetics of these agents will aid in assessing anti-inflammatory concentrations. Although NSAID almost fully block PG synthesis, they are only rarely associated with deleterious effects and this points to a generally pathological role for PGs. A good correlation between the capacity of a variety of chemicals to inhibit PG synthesis in vitro and to supress inflammation in the rat paw edema model (Ham et al 1972) points to this as the mechanism of anti-inflammatory action. The correlation between optical isomers is particularly convincing in this regard. NSAID also have a high affinity for albumin and this also correlates closely with the efficiency of these compounds as anti-inflammatory agents (Gryglewski 1974).

Finally, anti-inflammatory glucocorticoids block PG synthesis in cells (Weissmann 1980), among other actions, and this provides further evidence for a central role of PGs in inflammation.

3.2 Benzoic Acid Analogs and Inflammation: Problems Examined

To study the effects of the benzoic acid analogs in inflammation, the following questions were addressed:

1. In studies of the benzoic acid analogs in the rat paw carrageenin-induced edema model, agents such as ASA and 2-PBA, which inhibit platelet PG synthesis, displayed anti-inflammatory activity however 3-MP and ABA, which were weak platelet PG synthesis inhibitors, also had anti-inflammatory activity. 3-PBA actually increased inflammation in this model. Can the effects of these agents be reflected in the effects on PG synthesis from another tissue source related to inflammation such as polymorphonuclear, neutrophilic leukocytes (PMNs) which have been reported to produce PGs or is the anti-inflammatory effect mediated through another mechanism not related to PGs?

2. Can the effects of these agents on inflammation be reflected in effects on PMN accumulation in vivo in the rat pleurisy model? This would increase our understanding of mechanisms of anti-inflammatory activity by implicating the PMN, a cell commonly found at sites of inflammation, in the mechanism of anti-inflammatory activity of NSAID.

3. Based on the report that ABA has anti-inflammatory activity but does not inhibit PG synthesis (Cerskus 1978), is this agent a possible alternative to ASA in that it is not associated with the inhibition of platelet function?

3.3 Effects of Benzoic Acid Analogs on Polymorphonuclear Leukocytes In Vivo

3.3.1 Effects of Benzoic Acid Analogs on Arachidonic Acid Metabolism by Human Peripheral Polymorphonuclear Leukocytes

3.3.1.1 Introduction

Prostaglandin (PG) production by polymorphonuclear leukocytes (PMNs) was first reported by Higgs and Youlten (1972) who used glycogen elicited cells from the rabbit peritoneal cavity. Killed bacteria (Pertussis vaccine) increased the PG production by these cells. McCall and Youlten (1973) reported that the PGE_1 produced by these cells had chemotactic activity and the synthesis of PGE_1 was inhibited by indomethacin. Higgs et al (1976) reported that these cells converted PGG_2 and PGH_2 to TXA_2 and that homogenates of phagocytosing PMNs produced a mixture of TXA_2 and stable PGs while resting cell homogenates did not.

Wentzell and Epand (1978) reported that rat peritoneal exudate PMNs produced PGs and they pointed to the coincident rise in PMN cell number and PGs in inflammatory exudates. Di Rosa and Persico (1979) demonstrated that steroidal drugs inhibited PG production by these cells and Dray et al (1980) demonstrated that NSAID inhibited the PG synthesis capability.

Zurier and Sayadoff (1975) were the first to report that stimulated

human peripheral PMNs produced PGE_1 and $PGF_{1\alpha}$. Zymosan particles, which consist of a mixture of polysaccharides, proteins and ash derived from yeast cell walls or the entire yeast cell, were used to stimulate the PMNs. Goldstein et al (1978) found that resting human peripheral PMNs produced little TXA_2 but the amount increased 10 fold upon addition of serum treated zymosan (STZ). Zymosan, being an "anticomplementary" substance, becomes coated with the third component of complement (C3) upon incubation in serum. The "opsonic" or phagocytosis promoting fragment of complement (C3b) now available on the zymosan particles stimulated specific receptors on PMNs to activate the cells and to induce phagocytosis. Goldstein et al (1975) reported that STZ in buffer was readily agglutinated by rabbit antibody to human C3, confirming that fragments of C3 were bound to the zymosan. Upon exposure to STZ, human PMNs generate superoxide anion and release lysosomal enzymes and AA. The studies of Goldstein et al (1978) were based on the work by Smolen and Shohet (1974), showing that the phospholipid of phagocytosing PMNs had a marked decrease in AA content. Goldstein et al (1978) demonstrated that not only hydrocortisone but also superoxide dismutase, an enzyme which converts $O_2^{\cdot -}$ to H_2O_2 and O_2, blocked TXA_2 production thus oxygen centred free radicals were involved in PMN TXA_2 production.

Stenson and Parker (1979) also found that human PMNs produced PGE_2, HHT and TXA_2. They reported however that, compared to the lipoxygenase products reported by Borgeat et al (1976) and Borgeat and Samuelsson (1979a), these cyclo-oxygenase products were minor in quantity. These workers also suggested that some or all of the cyclo-oxygenase products in human peripheral PMN preparations may have resulted from contaminating platelets.

Bonser et al (1981a) reported that the human promyelocyte cell line HL60, when induced to differentiate into mature granulocytes with dimethylsulfoxide (DMSO), produced the cyclo-oxygenase derived HHT and other products.

Other leukocytes have been reported to produce PGs. Humes et al (1977) reported that macrophages produced PGs in response to zymosan. PGE_2 was the major PG produced and TXB_2 was the minor one. This work suggested that care must be taken in assigning the source of PG production to PMNs when using cells from animal inflammatory exudates, as macrophages are commonly found here. Kojima et al (1980) reported large conversions of AA to PGD_2 by rat bone marrow and Steinhoff et al (1980) reported that rat basophilic leukemia cells produced large amounts of PGD_2 enzymatically.

In other systems, Blackwell et al (1978) found that whole blood produced 6-keto $PGF_{1\alpha}$ and this was attributed to leukocytes. Viinikka and Vlikorkala (1981) were unable to block the production of 6-keto $PGF_{1\alpha}$ in this system after ASA ingestion by the human volunteers. Although 6-keto $PGF_{1\alpha}$ has been found in inflammatory exudates, the concentration has been reported to decrease as the lesion progresses (Higgs and Salmon 1979). Also, Bombardiere et al (1981) found a lack of correlation between PGE_2 levels and PMN counts in the synovium.

All evidence concerning PG production by PMNs is still inconclusive due to such problems as platelet contamination and mixed cell populations. Conflicting reports on the levels of PGs in inflammatory exudates, as already stated, make it difficult to ascertain if the PMN is the source of the inflammatory PGs.

Concerning drug effects on PMNs, current evidence suggests that

although indomethacin, at high concentrations, blocks lysosomal enzyme release from STZ stimulated rabbit PMNs, ASA, at doses up to 5mM has no effect on beta-glucuronidase or lysozyme release (Smolen and Weissmann 1980). From this work it was concluded that indomethacin works through mechanisms other than cyclo-oxygenase inhibition. Evidence does exist that NSAID block the migration of PMNs to sites of inflammation (see 3.4.1).

It was of interest to investigate the effects of the ASA-like drugs on PG production by PMNs - if, indeed, it existed. To date it appears that the correlation between anti-inflammatory activity and antiPG synthesis activity, at least in platelets, does not hold with these analogs (Cerskus and Philp 1981) as ABA and 3-MP had anti-inflammatory activity without inhibiting platelet PG synthesis. Differences in the sensitivities of cyclo-oxygenases, from different tissue sources, to ASA have been reported (see 2.4.1) and could be an explanation for this apparent divergence.

3.3.1.2 **Methods**

Preparation of Purified PMNs

Human peripheral PMNs were isolated using a method based on that of Ferrante and Thong (1978). This method is a modification of the Hypaque-Ficoll method and results in the separation of mononuclear and polymorphonuclear cells into 2 distinct bands. The separation medium consisted of a solution of 8.2% Ficoll 400 (Sigma Chemical Co., St. Louis) and 17% sodium diatrizoate (Sigma Chemical Co., St. Louis) at pH 7.4. Whole blood was citrated as described in the platelet studies. The separation medium (3ml) was placed into 15ml siliconized conical

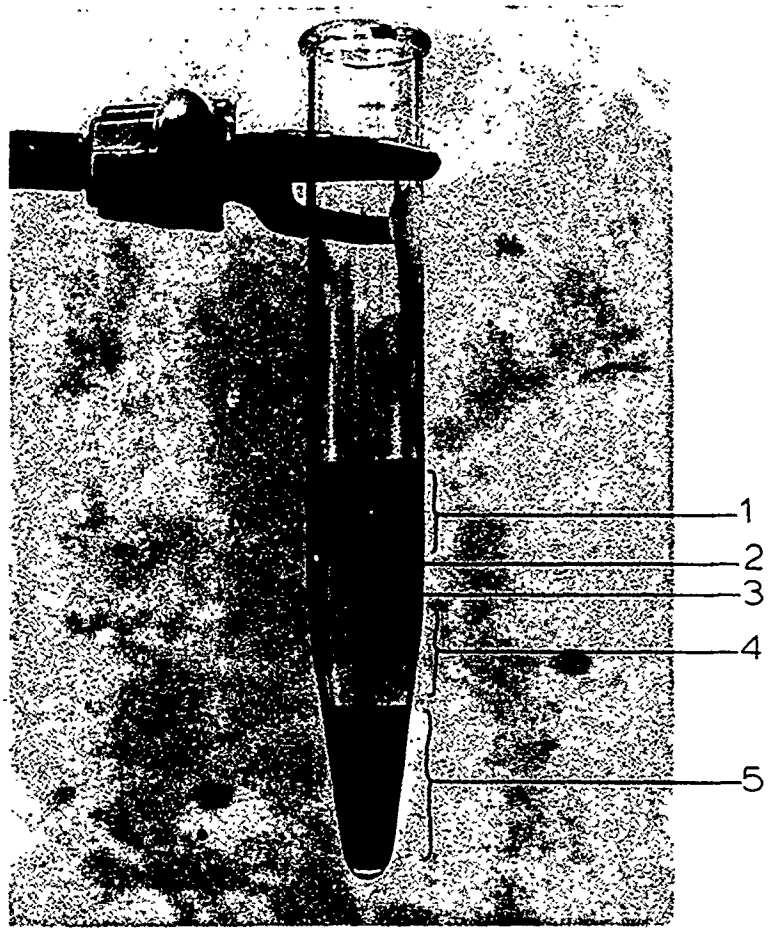
centrifuge tubes using a glass pipette and whole citrated blood (3ml) was carefully layered on top of the separation medium using a glass pipette. Positioning the pipette 1-2 cm from the top of the medium, blood was allowed to run down the side of the test tube slowly, as the pipette was gradually withdrawn from the top of the medium. The tubes were spun in a clinical centrifuge at 200 x g for 20 minutes (Plate 3). In some experiments, the top layer of plasma and the top cell layer of mononuclear cells were removed by means of a Pasteur pipette connected with tubing to a vacuum flask. The PMN layer and the rest of the medium down to the red cell layer was removed with a Pasteur pipette and placed into a 10ml siliconized test tube. To this was added equal volumes of saline and the tube was centrifuged at 968 x g for 10 minutes to produce a PMN pellet. The PMNs were then resuspended, using a Pasteur pipette, into 5ml of saline and centrifuged again as above. This process was repeated one more time to ensure adequate washing of the cells. The PMNs were suspended in Dulbecco's phosphate buffered saline (PBS) (Appendix III) and all assays were carried out in this buffer.

In order to obtain PMN samples with less platelet contamination, PMNs were isolated by centrifugation as above. The plasma, which made up the top layer, was removed and centrifuged at 968 x g for 20 min to remove any further cells. The PMN layer was removed as described above, saline was added and the cells were centrifuged at 968 x g for 20 minutes. The PMN pellet was resuspended in the cell free plasma and once again subjected to centrifugation on the Ficoll-sodium diatrizoate medium. The PMN band was accompanied by an upper buffy layer found to consist of platelets. This upper layer was removed using a Pasteur pipette and the PMNs were washed as before. For most of the studies, the

Plate 3 Separation of human peripheral polymorphonuclear neutrophilic leukocytes (PMNs).

Whole blood (3 ml) was carefully layered onto 3 ml of sodium diatrizoate-Ficoll 400 medium and spun at 200 x g for 20 min.

1. Plasma containing platelets
2. Plasma/medium interface containing mononuclear cells and platelets
3. Cell free area
4. Medium containing PMNs and platelets (1:1)
5. Red cells


1
2
3
4
5

latter PMN preparation was used.

Cells were counted using improved Neubauer Hemocytometers (for white cell dye see Appendix III). Cell purity was assessed by cell smears on microscope slides stained with Wright's stain (Appendix III). Cell viability was assessed using the trypan blue exclusion test (Appendix III). The above methods did not appear to adversely affect cell viability. Platelet contamination was measured using the platelet counting technique already described.

Preparation of Serum Treated Zymosan (STZ)

STZ was prepared according to Goldstein et al (1975). Fresh serum was prepared from whole blood obtained as described above but without the addition of citrate. Whole blood (8ml) was placed into a test tube and left to stand at 4° C for 18 minutes. Coagulated blood, adhering to the wall of the test tube was then removed from the wall by running a small round wooden stick around the inside wall to a depth of 1.5 cm. The blood was allowed to stand for a further 20 minutes after which the "cleaning" procedure was repeated and the blood was spun at 968 x g for 20 minutes. The serum supernatant (3-4ml) was removed using a Pasteur pipette and placed into a 10ml test tube.

Zymosan A (Sigma Chemical Co., St. Louis) was suspended in 6ml saline in a 10ml beaker and evaporated down by boiling to a volume of 2ml. The contents were placed into 2 Eppendorf Microtest tubes and centrifuged for 2 minutes. The supernatant was removed and the boiled zymosan pellet was resuspended in 1.5ml fresh serum and incubated for 30 minutes at 37° C. The contents were spun for 2 minutes, the zymosan was washed twice in saline and the zymosan was suspended in PBS at a final

concentration of 8-40 mg/ml.

Incubation Procedure

PMNs were divided into portions containing 1-5 x 10^7 PMNs per sample in a volume of 500ul. Cells were incubated at 37° C with the addition of 50ul PBS or drug made up in PBS for a period of 7 minutes. ^{14}C-AA (200ul, 30uM, specific activity 6 uCi/umole) was added with PBS 250ul or STZ made up in PBS (250ul), the test tube contents mixed by a brief vortex and the incubation was allowed to proceed for various times. The extraction of the PGs and TLC separation were the same as reported for platelets. The % conversion of ^{14}C-AA to material co-chromatographing with PGs was 5% in STZ-stimulated cells and less than 1% in nonstimulated cells (see Appendix IV).

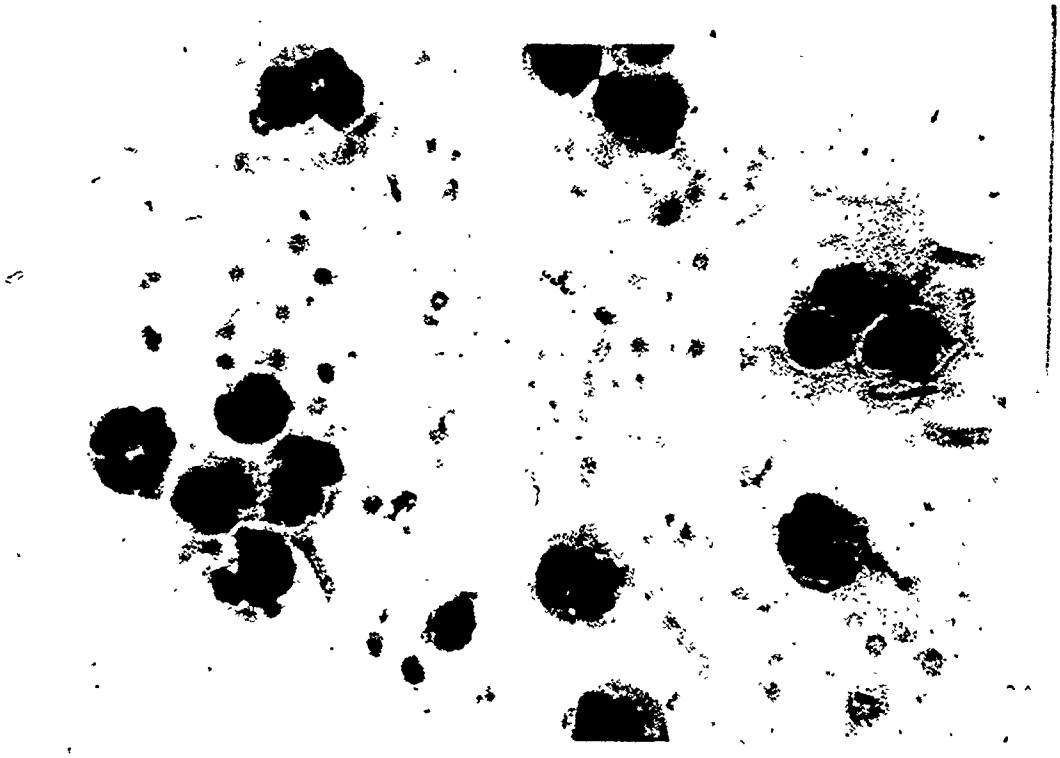
In some experiments, the products remaining on the column after elution with 6% MeOH/$CHCl_3$ were eluted with 2-6ml 100% methanol into a plastic scintillation vial and dried under a stream of air. Methanol, 500ul, was added to the vial to dissolve the product and 10ml of counting fluid were added. In one experiment the methanol fraction was dried under nitrogen and redissolved in 10ul ethanol. This was spotted on a silica gel G plate (Eastman Chromogram) along with small amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Sigma Chemical Co., St. Louis). The plate was developed once under saturating conditions using methanol/chloroform/water (65:25:4, v:v) and the spots were stained with iodine vapour.

3.3.1.3 **Results**

Using these cell separation methods, PMNs always made up more than 98% of the white cell population (Plate 4) and cell viability assessed using the trypan blue exclusion test, was always greater than 90%.

Plate 4 Photomicrograph of PMNs after separation from human whole blood.

Cells were separated from whole blood, washed with saline and resuspended in human blood serum. A typical blood-type smear of this cell suspension was done and stained with Wright's stain to visualize the cells. x 1000.



The profiles of products of the 6% MeOH/$CHCl_3$ fraction of silicic acid column chromatography of the enriched PMN mixtures were examined by TLC. In early studies, cells were subjected to only one separation treatment and this accounts for the large cell number available for study. In Fig. 29A the effect of STZ on AA metabolism by PMNs is demonstrated. STZ (10 mg/ml) caused large increases in the PGD_2 and TXB_2 peaks and this is a typical profile of AA metabolism by PMNs. Peaks of activity from stimulated cells were inhibited by 180uM ASA. The methanol fractions from silicic acid columns of stimulated cells contained large amounts of polar products compared to nonstimulated cells (Fig. 29A). These products were not inhibited by ASA and, even nonstimulated PMN levels were higher than was found for platelets.

Early studies of the effects of the ASA-like drugs on the metabolism of AA by this PMN enriched cell mixture revealed patterns of inhibition similar to those reported for platelets. For this reason, and also based on literature reports of the presence of platelets in this preparation, the contamination of PMNs by platelets and the platelet contribution to the observed 6% MeOH/$CHCl_3$ profile were examined. Platelets were found to contaminate PMN samples in the ratio of 1:1. The actual contribution of equal numbers of platelets to the 6% MeOH/$CHCl_3$ profile is shown in Fig. 29B. From this data it is evident that:

i) the peak of activity corresponding to TXB_2 in the nonstimulated cells can almost totally be accounted for by platelets

ii) small numbers of platelets produce a major peak corresponding to PGE_2 under these conditions although PGD_2 and TXA_2 are also major

iii) STZ does not cause an increase in platelet AA metabolism but rather changes the profile by causing a decrease in the size of the PGD_2 and

Fig. 29 Metabolism of ^{14}C-AA by PMNs and platelets I.

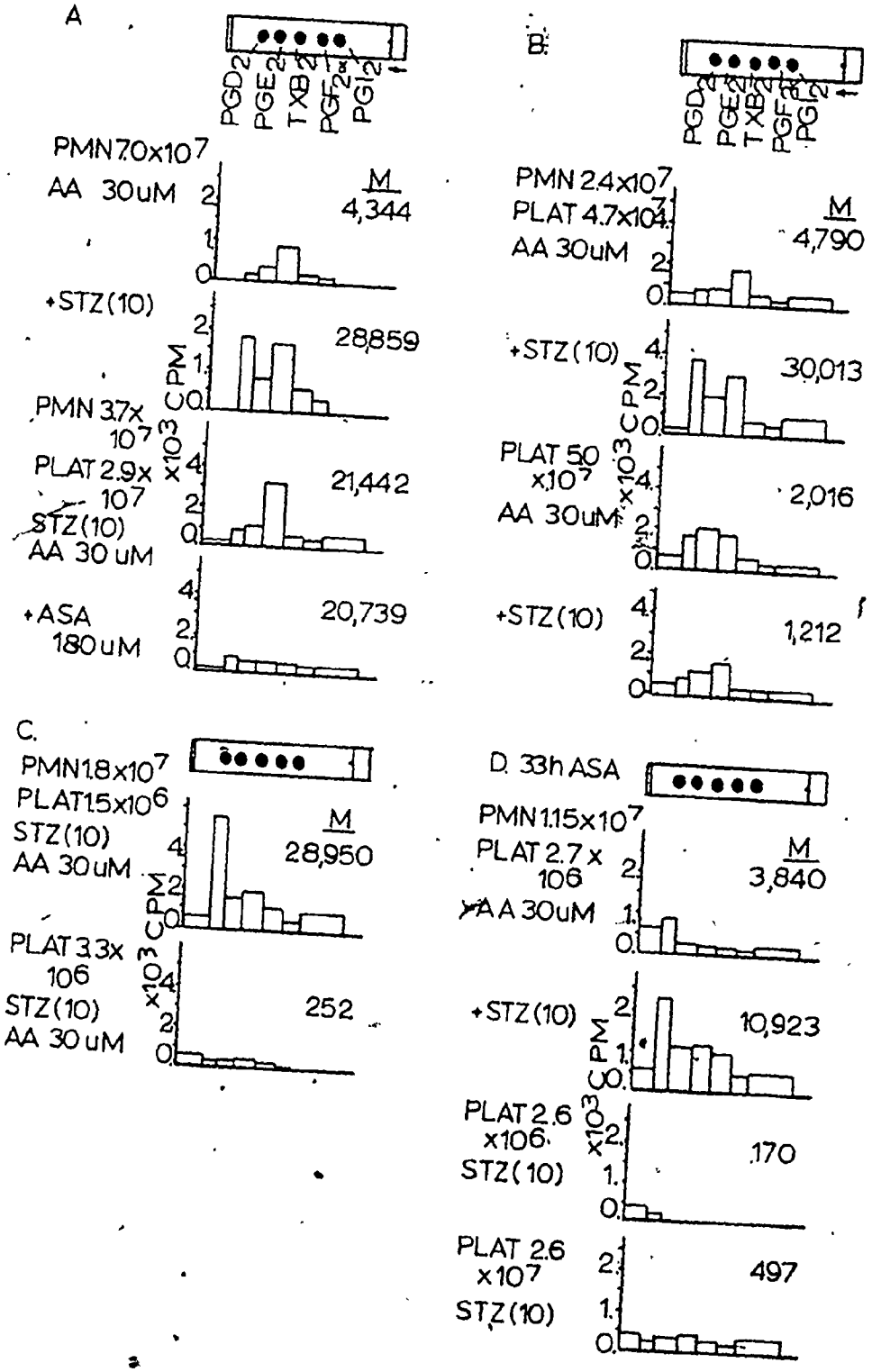
Typical TLC profiles of ^{14}C-AA products of the 6% MeOH/$CHCl_3$ silicic acid column fraction of the chloroform extracts of the following reaction mixtures. Solvent moves in the direction of the arrow. PG standards are labelled. Plates were cut up and counted by liquid scintillation counting. M is the CPM of the 100% MeOH silicic acid column fraction corresponding to ^{14}C-AA products that are more polar than PGs.

A. Nonstimulated PMNs (containing platelets); plus STZ (10 mg/ml); PMNs with platelet contamination and stimulated with STZ (10 mg/ml); plus ASA (180 uM).

B. Platelet-PMN mixture, nonstimulated; plus STZ (10 mg/ml); similar number of platelets without PMNs; plus STZ (10 mg/ml).

C. Platelet-PMN mixture with reduced platelet number and STZ (10 mg/ml); platelets without PMNs plus STZ (10 mg/ml).

D. PMN-platelet control from donor after ASA (650 mg) 33 hr previously; plus STZ (10 mg/ml); similar number of platelets without PMNs, with STZ (10 mg/ml); 10 fold increase in platelets without PMNs, with STZ (10 mg/ml).


A
PGD2
PGE2
TXB2
PGF2α
PGI2
PMN 7.0x10^7
AA 30uM
M
4,344
+STZ(10)
28,859
PMN 3.7x 10^7
PLAT 2.9x 10^7
STZ(10)
AA 30 uM
21,442
+ASA 180uM
20,739
B.
PMN 2.4x10^7
PLAT 4.7x10^7
AA 30uM
M
4,790
+STZ(10)
30,013
PLAT 50 x10^7
AA 30uM
2,016
+STZ(10)
1,212
CPM
x10^3
C.
PMN 1.8x10^7
PLAT 1.5x10^6
STZ(10)
AA 30uM
M
28,950
PLAT 3.3x 10^6
STZ(10)
AA 30uM
252
D. 33h ASA
PMN 1.15x10^7
PLAT 2.7 x 10^6
AA 30uM
M
3,840
+STZ(10)
10,923
PLAT 2.6 x10^6
STZ(10)
170
PLAT 2.6 x10^7
STZ(10)
497

PGE_2 peaks leaving TXB_2 as the major peak.

Therefore platelets did contribute significantly to the PMN profile, although not totally. STZ stimulated platelets did not account for the increase in products found in the methanol fraction of PMN incubates and did not contribute significantly to these products.

In order to eliminate the high level of platelet contamination in PMNs, the cells were subjected to the separation procedure twice (3.3.1.2). Samples were obtained in which the platelet contamination was reduced anywhere from 1:4 to 1:10 (platelet:PMN). Under these conditions the platelet contribution to PMN AA metabolism was minimized (Fig. 29C). The separation procedure did not adversely affect the PMNs as:

i) cell viability was normal based on the trypan blue exclusion test

ii) platelets isolated under similar conditions produced typical patterns of AA metabolism

iii) the components of the separation medium did not inhibit platelet aggregation

iv) PMNs remained sensitive to STZ with regard to increased polar metabolite production as found in the methanol fraction. This value was related to the cell number in the incubation medium.

Further attempts were made to confirm that the PMNs were the source of the AA products by using blood from a donor who had ingested 650 mg ASA 33 hours prior to donation. In these experiments the assumption was made that ASA, which irreversibly blocks the cyclo-oxygenase enzyme, would inactivate all but the new platelets (10-20%) while PMNs, being nucleated cells, could synthesize new enzyme in this time period. Under these conditions the typical profile of the 6% MeOH/$CHCl_3$ products from STZ stimulated PMNs was seen (Fig. 29D) while PG production, by equal

numbers of platelets and a 10 fold increase in that number, were negligible (Fig. 29D).

The major peaks of ^{14}C-AA metabolites produced by PMNs co-chromatographed with PGD_2 and TXB_2. To confirm that these products were cyclo-oxygenase in origin, PMNs were incubated with high concentrations of indomethacin to inhibit their synthesis (Fig. 30A). At concentrations as high as 90uM indomethacin did not inhibit the synthesis of these metabolites nor the polar metabolites found in the methanol fraction. This suggested that the metabolites of AA were not cyclo-oxygenase in origin. Various changes in conditions were made in order to allow for optimal cyclo-oxygenase activity and these included lowering the substrate concentration to 10uM, varying incubation times with substrate and inhibitors and omitting the STZ stimulus. Under no conditions was ASA inhibitory to any of the products. Based on reports that adrenaline and reduced glutathione increased cyclo-oxygenase activity, that adrenaline inhibited lipoxygenase activity and that, in combination, adrenaline and reduced glutathione caused maximal PGD_2 production (Jakschik et al 1980), these agents were examined in the PMN incubates (Fig. 30B). Adrenaline (1.2mM) inhibited all peaks, leaving a typical PMN profile at much reduced levels. Reduced glutathione had no marked effect. Together a slight reduction in activity was seen. These results suggest that the products are not cyclo-oxygenase in origin. When used in combination, glutathione prevented the oxidation of adrenaline to adrenchrome (the sample did not turn pink) and this suggests that the oxidation of adrenaline plays a role in inhibiting the oxidation of the AA.

Nordihydroguaiaretic acid (NDGA), an inhibitor of both lipoxygenase

Fig. 30 Metabolism of ^{14}C-AA by PMNs and platelets II.

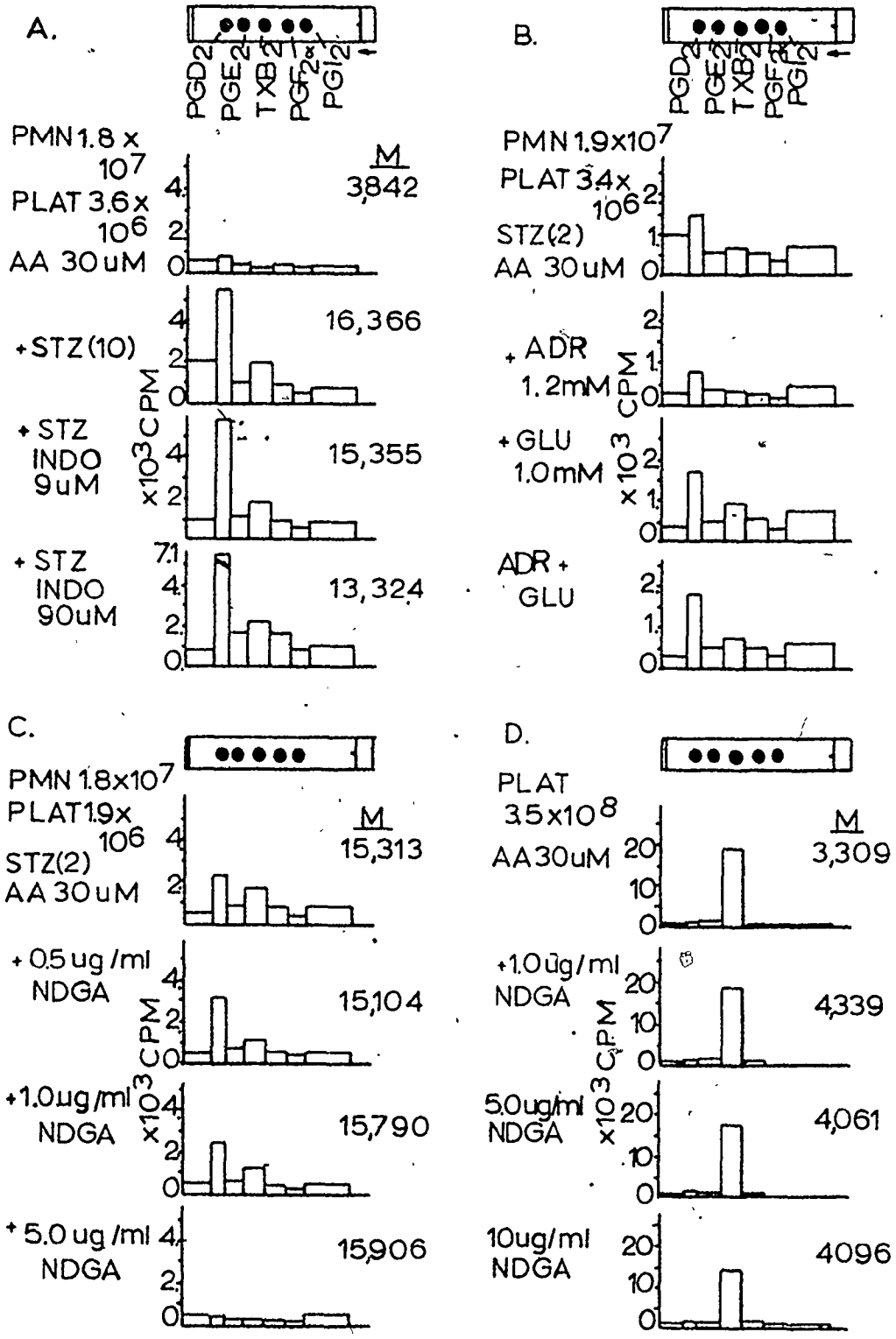
Typical TLC profiles of ^{14}C-AA products of the 6% MeOH/$CHCl_3$ silicic acid column fraction of chloroform extracts of the following reaction mixtures. Solvent moves in the direction of the arrow. PG standards are labelled. Plates were cut up and counted by liquid scintillation counting. M is the CPM of the 100% MeOH silicic acid column fraction corresponding to ^{14}C-AA products that are more polar than PGs.

A. Control PMNs; plus STZ (10 mg/ml); plus STZ (10 mg/ml) and indomethacin (9 uM); plus STZ (10 mg/ml) and indomethacin (90 uM).

B. Control PMNs plus STZ (2 mg/ml); plus 1.2 mM adrenaline; plus 1.0 mM reduced glutathione; plus adrenaline and glutathione.

C. Control PMNs plus STZ (2 mg/ml); plus 0.5 ug/ml NDGA; plus 1.0 ug/ml NDGA; plus 5.0 ug/ml NDGA.

D. Control platelets alone; plus 1.0 ug/ml NDGA; plus 5.0 ug/ml NDGA; plus 10.0 ug/ml NDGA.


A.
PGD2
PGE2
TXB2
PGF2α
PGI2
PMN 1.8 x 10^7
PLAT 3.6 x 10^6
AA 30 uM
M
3,842
+ STZ (10)
16,366
+ STZ INDO 9 uM
15,355
+ STZ INDO 90 uM
13,324
CPM
x10^3
B.
PMN 1.9x10^7
PLAT 3.4x 10^6
STZ(2)
AA 30 uM
+ ADR 1.2mM
+ GLU 1.0mM
ADR + GLU
C.
PMN 1.8x10^7
PLAT 1.9x 10^6
STZ(2)
AA 30 uM
M
15,313
+ 0.5 ug/ml NDGA
15,104
+1.0 ug/ml NDGA
15,790
+ 5.0 ug/ml NDGA
15,906
D.
PLAT 3.5x10^8
AA 30 uM
M
3,309
+1.0 ug/ml NDGA
4,339
5.0 ug/ml NDGA
4,061
10 ug/ml NDGA
4096

and cyclo-oxygenase but more specific for the lipoxygenase enzyme at low doses (Morris et al 1980), caused a dose dependent inhibition of these products with maximal inhibition at a concentration of 5 ug/ml (Fig. 30C). This dose did not affect the polar methanol metabolites. NDGA did not block PG production in freeze thawed platelet lysates, but at 10 ug/ml had a weak effect on intact platelets incubated under conditions similar to the PMN (Fig. 30D). The chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-7}M), caused an increase in 6% MeOH/$CHCl_3$ products without affecting the polar metabolites in the methanol fraction. Carrageenin (2 mg/ml) had no effect on the production of AA metabolites by PMNs (not shown).

The effect of 3 rapid freeze thaws on PMN AA metabolism was examined using the original substrate concentration of 30uM (Fig. 31A). This procedure is used to prepare freeze thawed platelet lysates. Total metabolite production was drastically reduced although a major peak of activity co-chromatographed with PGD_2. Again, ASA did not inhibit this peak. STZ (10 mg/ml) did not increase the amount of metabolites suggesting that intact cells were required for STZ stimulation of PMN AA metabolism. The incorporation of AA into polar metabolites in the methanol fraction was also drastically reduced.

The effects of the ASA-like drugs and ETYA (5,8,11,14-eicosatetraynoic acid, an inhibitor of cyclo-oxygenase and lipoxygenase activity) were examined on the PMN AA metabolites. ETYA (35uM) inhibited all peaks of metabolites in the 6% MeOH/$CHCl_3$ fraction and also blocked the production of the more polar metabolites (Fig. 31B). 2-PBA, 3-PBA and ABA did not have any major effects (Fig. 31B,C). 3-MP (500uM) caused a shift in the peaks of metabolites (Fig. 31C). 3HMP (250uM) inhibited

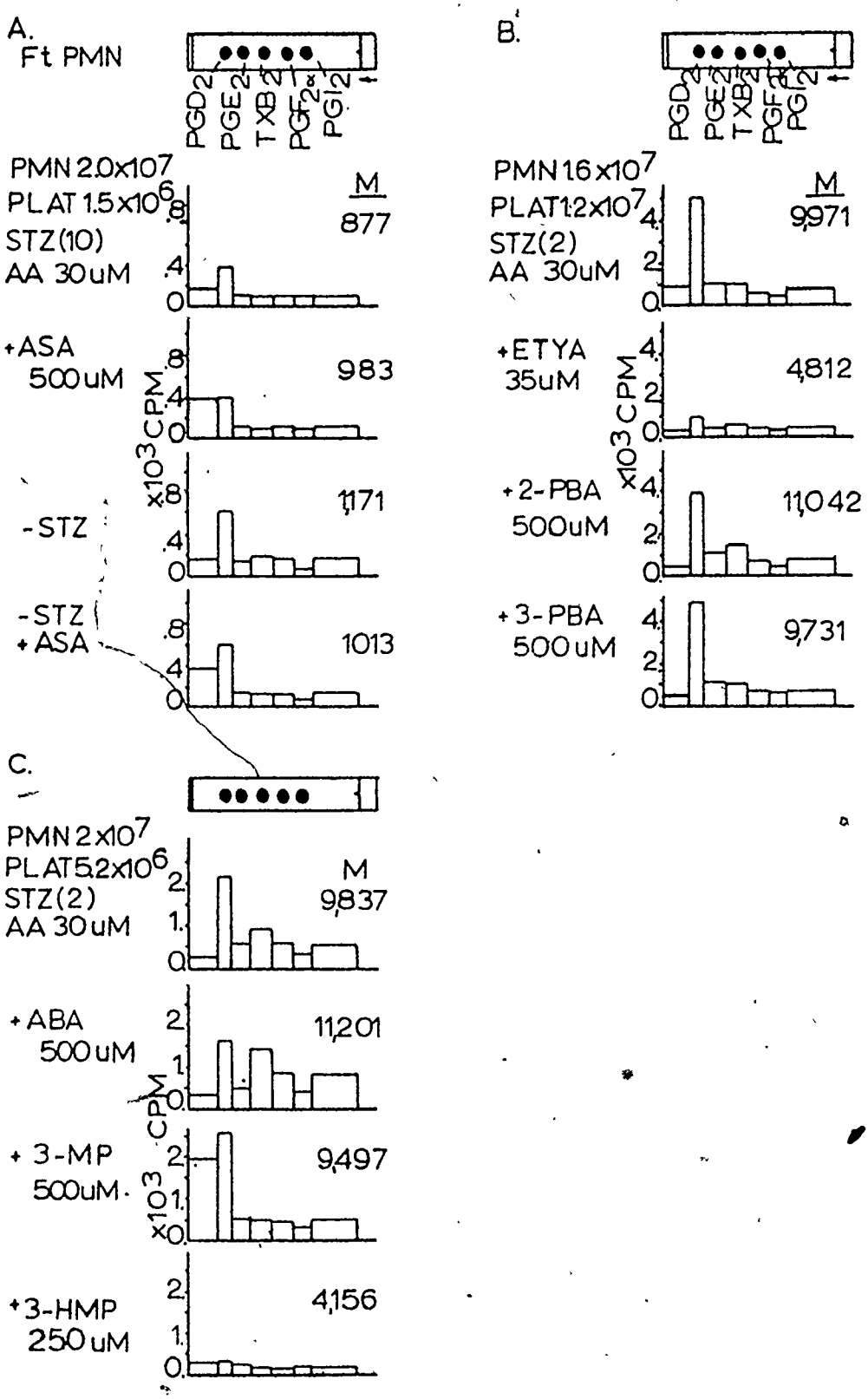
Fig. 31 Metabolism of ^{14}C-AA by PMNs and platelets III.

Typical TLC profiles of ^{14}C-AA products of the 6% MeOH/$CHCl_3$ silicic acid column fraction of chloroform extracts of the following reaction mixtures. Solvent moves in the direction of the arrow. PG standards are labelled. Plates were cut up and counted by liquid scintillation counting. M is the CPM of the 100% MeOH silicic acid column fraction corresponding to ^{14}C-AA products that are more polar than PGs.

A. Control PMNs that have been freeze thawed (Ft) three times and stimulated with STZ (10 mg/ml); with ASA (500 uM); without STZ; without STZ but with ASA (500 uM).

B. Control, intact PMNs with STZ (2 mg/ml); with ETYA (35 uM); with 2-PBA (500 uM); with 3-PBA (500 uM).

C. Control PMNs with STZ (2 mg/ml); with ABA (500 uM); with 3-MP (500 uM); with 3HMP (500 uM).


A.
Ft PMN
PGD2
PGE2
TXB2
PGF2α
PGI2
PMN 2.0x10^7
PLAT 1.5x10^6
STZ(10)
AA 30uM
M
877
+ASA
500uM
983
-STZ
1171
-STZ
+ASA
1013
x10^3 CPM
B.
PMN 1.6x10^7
PLAT 1.2x10^7
STZ(2)
AA 30uM
9971
+ETYA
35uM
4812
+2-PBA
500uM
11042
+3-PBA
500uM
9731
C.
PMN 2x10^7
PLAT 5.2x10^6
STZ(2)
AA 30uM
9837
+ABA
500uM
11201
+3-MP
500uM
9497
+3-HMP
250uM
4156
CPM
x10^3

all the 6% MeOH/$CHCl_3$ peaks and, unlike NDGA, inhibited the production of the more polar metaboiltes. The effects of ASA on this profile were negligible.

Some of the data appeared contradictory concerning the nature of the AA metabolites. Using the regular solvent system (II) (ethyl acetate/acetic acid/isooctane/water, 110:20:50:100, v:v) these products co-chromatographed with PGD_2 and TXB_2 and were inhibited by 3HMP, an agent previously shown to be a potent PG synthesis inhibitor. On the other hand the metabolites were not inhibited by the usual NSAID, were not increased by adrenaline and reduced glutathione but were decreased by adrenaline and, finally, they were readily inhibited by NDGA at concentrations specific for the lipoxygenase enzyme system. Attempts were made to separate the peaks from the known standards using two other solvent systems (Fig. 32). In each case the peak of the products co-chromatographed with PGD_2 and a peak at TXB_2 was also apparent. The peak of ^{14}C-AA products was finally separated from the PGD_2 standard using reverse phase thin layer chromatography plates (Whatman MKC18F) and a solvent system of 26% acetonitrile in water at pH 3.0 (Fig. 33A).

To evaluate the nature of the more polar products the methanol fraction from the PMN incubate was dried down under nitrogen, redissolved and run on silica gel G plates as usually done for the 6% MeOH/$CHCl_3$ fraction. Using a solvent system commonly used for phospholipids (3.3.1.2), migration of the products occurred and corresponded to PE and PC standards. The majority of the products co-chromatographed with PE (Fig. 33B). This indicates that large amounts of exogenous AA are incorporated in the membrane phospholipids of STZ-stimulated PMNs.

Fig. 32 Typical TLC profiles of ^{14}C-AA products of the 6% MeOH/$CHCl_3$ silicic acid column fraction of chloroform extracts of PMN incubates using various TLC solvent systems.

Solvent moves in the direction of the arrow. PG standards are labelled. Plates were cut up and counted by liquid scintillation counting.

Solvent II: Ethyl acetate/acetic acid/isooctane/water (110:20:50:100, v:v) 2 developments.

Solvent I: Acetic acid/ethyl acetate (1:99, v:v) 2 developments.

Solvent III: Benzene/dioxane/acetic acid (20:10:1, v:v) 2 developments.

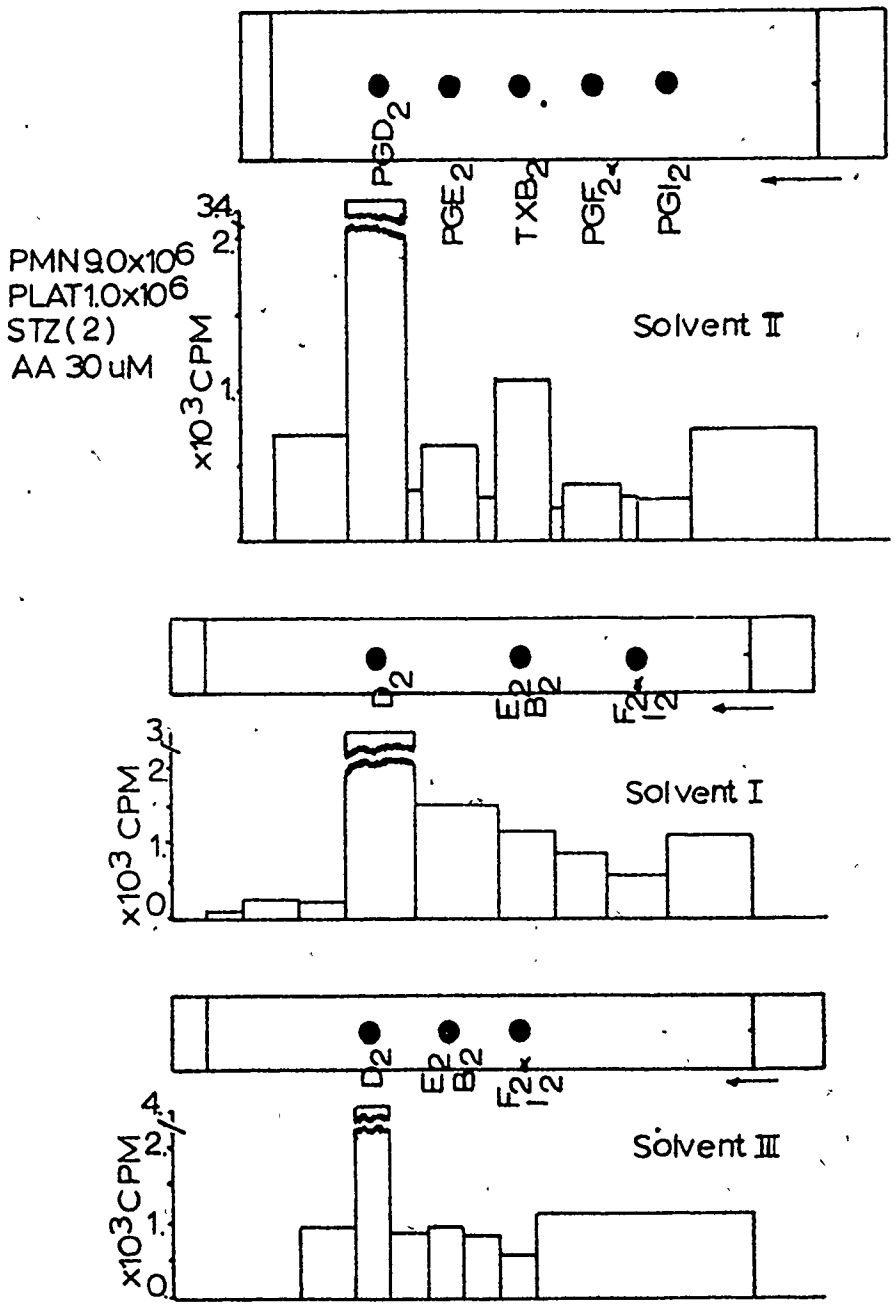
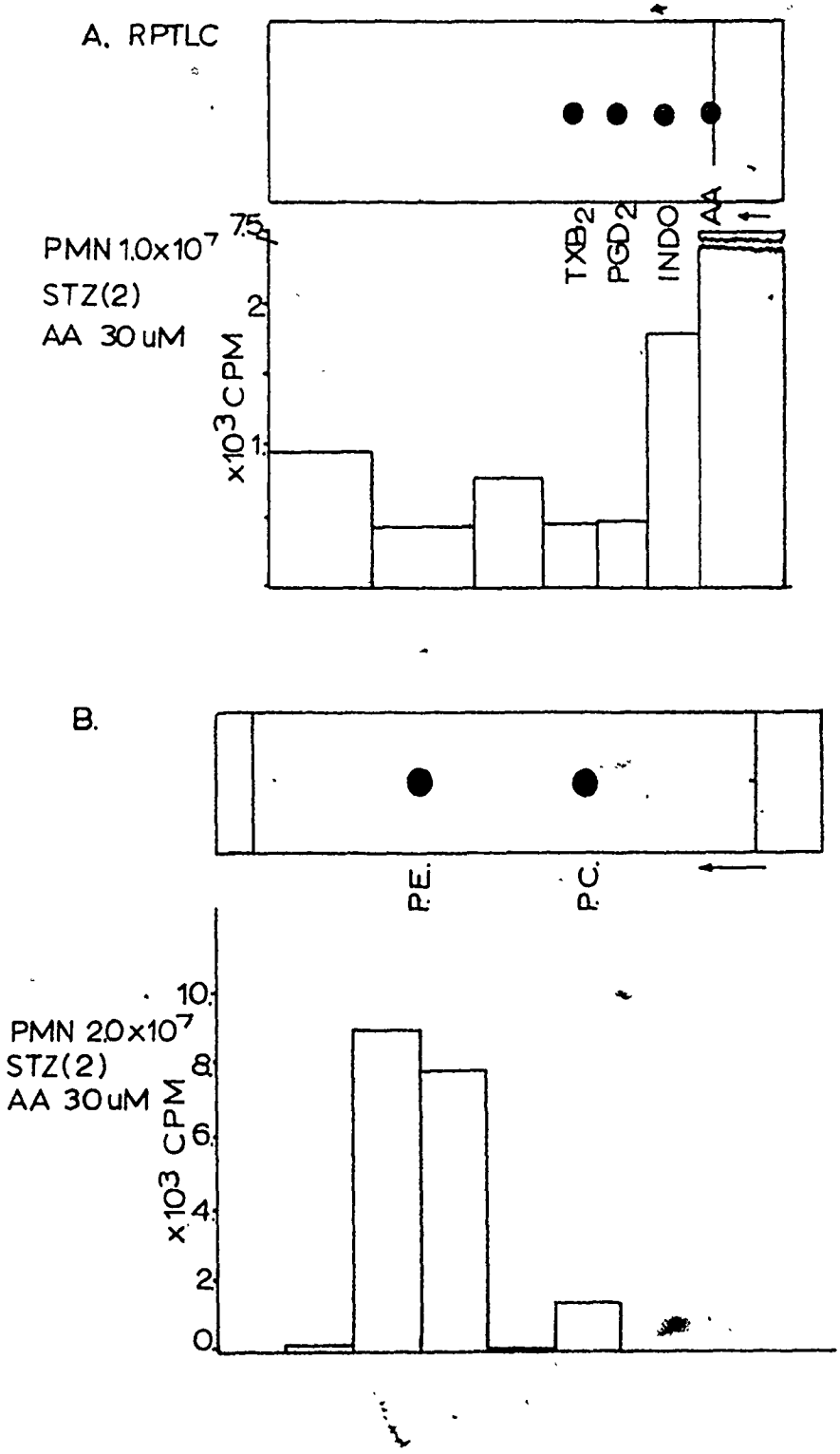

PMN 9.0x10^6
PLAT 1.0x10^6
STZ (2)
AA 30 uM
PGD2
PGE2
TXB2
PGF2α
PGI2
x10^3 CPM
Solvent II
Solvent I
Solvent III

Fig. 33 Typical TLC profiles of ^{14}C-AA products of PMNs.

Solvent flows in the direction of the arrow.

A. TLC of the 6% MeOH/$CHCl_3$ silicic acid column fraction of chloroform extracts of PMN incubates by reverse phase TLC using Whatman MKC18F plates and 26% acetonitrile in water (pH 3.0) as solvent. PG standards are labelled together with AA and indomethacin. Areas were scrapped from the plate and counted by liquid scintillation counting.

B. TLC of the 100% MeOH silicic acid column fraction (M) of chloroform extracts of PMN incubates using the regular plates (Eastman Chromogram) and a solvent system for phospholipids: methanol/chloroform/water (65:25:4, v:v).
PE = phosphatidylethanolamine
PC = phosphatidylcholine
Plates were cut up and counted by liquid scintillation counting.


A. RPTLC
TXB2
PGD2
INDO
AA
PMN 1.0x10^7
STZ(2)
AA 30 uM
x10^3 CPM
7.5
2
1
B.
P.E.
P.C.
PMN 2.0x10^7
STZ(2)
AA 30 uM
x10^3 CPM
10
8
6
4
2
0

The complete profile of products of ^{14}C-AA after incubation with PMNs was examined by omitting the silicic acid column separation step and applying incubate extracts directly to TLC plates (Fig. 34). Standards included the regular PGs as well as AA and indomethacin which, according to Jakschik et al (1980), co-chromatographs with 5,12-dihydroxy-eicosatetraenoic acid (5,12-diHETE, LTB_4), in this system. LTB_4 has potent biological activity (3.4.1.1). Most of the products, with the exception of those incorporated into phospholipid, moved with the indomethacin or further. This indicates that the metabolites of the 6% MeOH/$CHCl_3$ fraction are more polar than LTB_4 and are minor products of PMN AA metabolism. A peak of activity did correspond to PGD_2 and TXB_2.

Attempts were made to investigate further the possibility of PG synthesis by these cells by incorporating ^{14}C-AA into the PMN cell membrane. This was done based on the hypothesis that AA must be incorporated into specific phospholipid pools before labelled AA could "find" the cyclo-oxygenase enzyme. Cells were prelabelled by incubating with 3uM ^{14}C-AA (54.6 mCi/mmol) for 90 minutes then washed 3 times with saline. The labelled cells were resuspended in PBS and incubated at 37° C for 4 hours in the presence or absence of 500uM ASA. Chloroform extracts of the acidified incubation mixtures were run on 13 cm long thin layer plates. Peaks of products corresponding to PGs were minimal and not inhibited by ASA (Fig. 35). Peaks of radioactivity in the area of indomethacin were reduced to some extent and the amount of ^{14}C-AA incorporated into phospholipids was higher in the ASA sample. There did appear to be differences between the control and ASA in the peak corresponding to PGE_2. Because the control peak level was so low, the

Fig. 34 Typical TLC profiles of ^{14}C-AA products of chloroform extracts of PMN incubates without silicic acid column chromatography.

Solvent moves in the direction of the arrow. PG standards are labelled together with AA and indomethacin. Plates were cut up and counted by liquid scintillation counting.

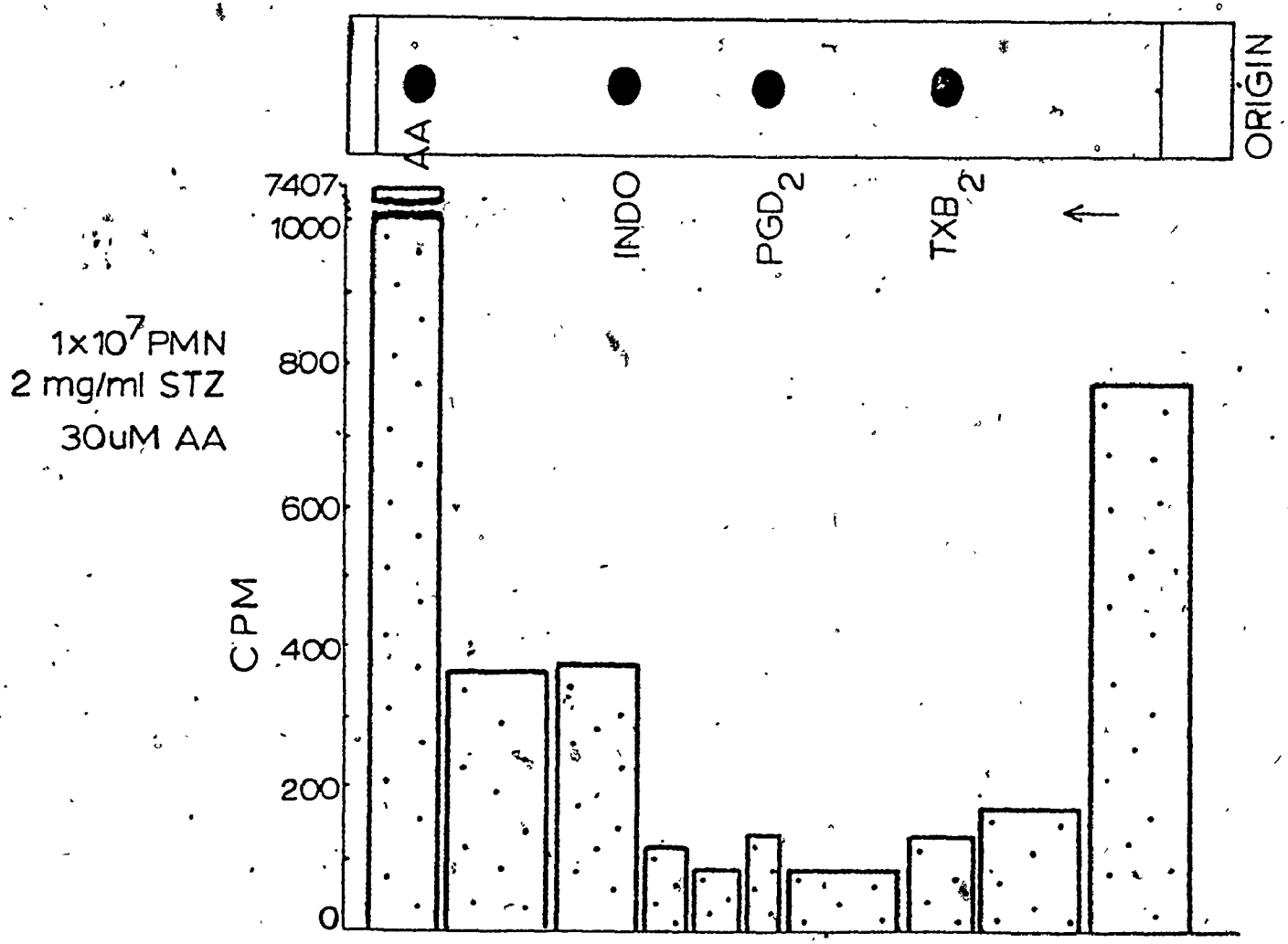
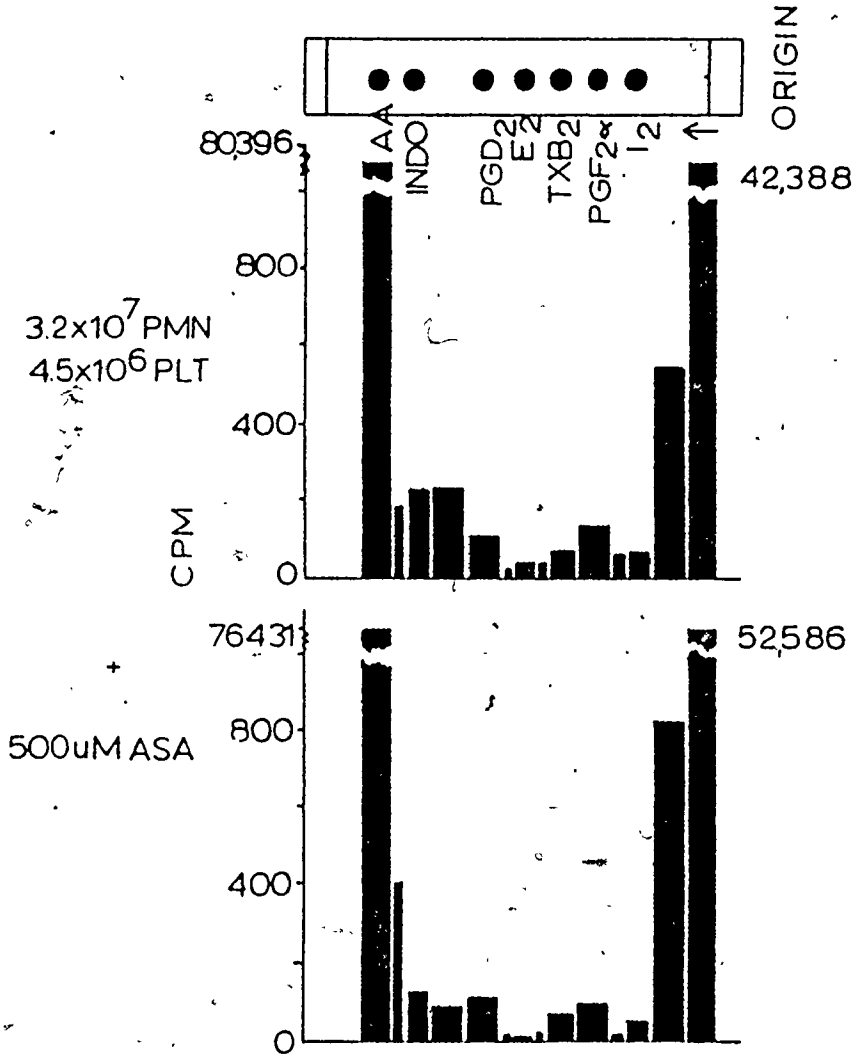

AA
INDO
PGD2
TXB2
ORIGIN
7407
1000
800
600
400
200
0
CPM
1x10^7 PMN
2 mg/ml STZ
30uM AA

Fig. 35 Typical TLC profiles of ^{14}C-AA products of the chloroform extracts of PMNs prelabelled with ^{14}C-AA (3 uM, 54.6 mCi/mmol) for 90 min.

Labelled cells were incubated at 37° C for 4 hours in the presence or absence of 500 uM ASA. Solvent moves in the direction of the arrow. PG standards are labelled as well as AA and indomethacin. CPM corresponding to AA and the origin are given. Plates were cut up and counted by liquid scintillation counting.


ORIGIN
AA
INDO
PGD2
E2
TXB2
PGF2α
I2
80,396
42,388
800
400
0
CPM
3.2x10^7 PMN
4.5x10^6 PLT
76431
52,586
800
400
0
500uM ASA

significance of the PG results are questionable, however, the cells for this experiment were isolated from a donor who had taken 600 mg ASA 24 hours earlier. Under these conditions, only about 10% of the platelets present are able to convert AA to cyclo-oxygenase products (10% of the platelets are replaced daily). The low level of platelets present, together with the fact that most platelets are not functional, eliminates them as a major contributor to this profile of AA products.

Early work using PMN preparations contaminated with equal numbers of platelets resulted in a peak of products co-chromatographing with TXB_2 which was inhibitable by NSAID. Platelets isolated alone and in equal numbers to those in the PMN suspension did not produce a major TXB_2 peak but rather a PGE_2 peak dominated. Repetition of these experiments using PMN populations containing equal numbers of platelets confirmed these results (Fig. 36A). The TXB_2 peak increased with STZ stimulation in the PMN suspension and was inhibited by 275uM ASA leaving a typical PMN profile. Equal numbers of platelets produced a peak at PGE_2 and this was reduced with no concomitant increase in TXB_2 upon addition of STZ. The profile of products produced by platelets appeared to depend also on the platelet number with a shift to increased TXB_2 with increased platelet number (Fig. 29B, 30D, 36A,B).

Further assessment of platelet-PMN interactions was made by adding increasing numbers of PMNs to platelet suspensions and resuspending the cells in a set volume. This large number of platelets alone produced a major peak corresponding to TXB_2 (Fig. 36B). Low numbers of PMNs resulted in increased PGD_2 and TXB_2 peaks. Logarithmic increases in the PMN number resulted in a steady decrease in the PGD_2 peak and finally a return to the original profile except for an increased TXB_2 peak. The

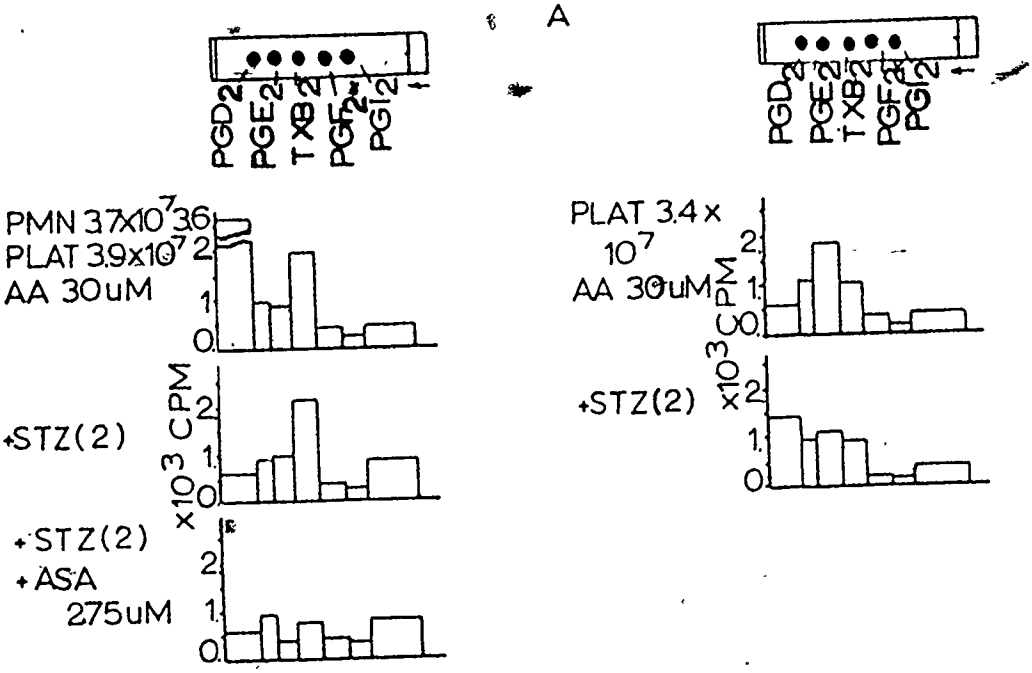
Fig. 36 The interaction of PMNs and platelets in the metabolism of ^{14}C-AA.

Typical TLC profiles of ^{14}C-AA products of the 6% MeOH/$CHCl_3$ silicic acid column fraction of chloroform extracts of the following reaction mixtures. Solvent moves in the direction of the arrow. PG standards are labelled. Plates were cut up and counted by liquid scintillation counting.

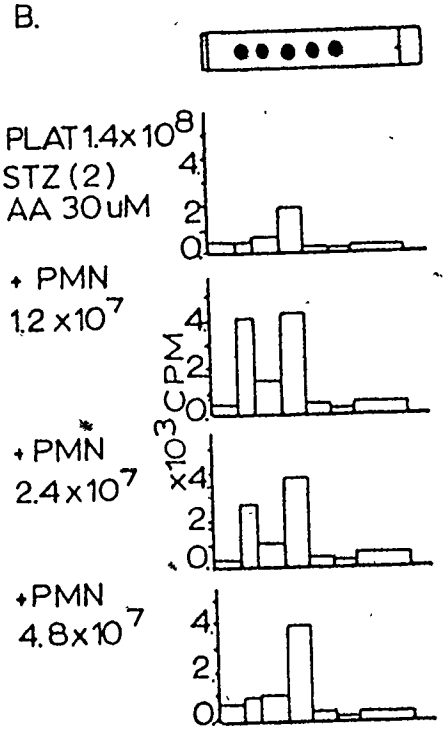
A. Control with 1:1 platelet:PMN ratio; with STZ (2 mg/ml); with ASA (275 uM); platelets alone; with STZ (2 mg/ml).

B. Control with only platelets in the reaction mixture and STZ (2 mg/ml); with 1.2×10^7 PMNs; with 2.4×10^7 PMNs; with 4.8×10^7 PMNs.

A


PGD2
PGE2
TXB2
PGF2α
PGI2
PMN 3.7X10^7 3.6
PLAT 3.9x10^7
AA 30uM
+STZ(2)
+STZ(2)
+ASA
275uM
PLAT 3.4 x
10^7
AA 30uM
+STZ(2)
x10^3 CPM

B.


PLAT 1.4x10^8
STZ (2)
AA 30uM
+ PMN
1.2 x10^7
+ PMN
2.4x10^7
+PMN
4.8x10^7
x10^3 CPM

amount of radioactivity incorporated into polar metabolites increased as the PGD_2 peak declined.

This work on platelet-PMN interactions can be summarized as follows:

(i) small numbers of platelets (less than 5×10^7/ml) alone convert exogenous AA mainly to PGE_2 and secondly to TXB_2. STZ actually decreases the levels of PGE_2 produced by platelets.

(ii) in the presence of PMNs, small numbers of platelets produce TXB_2 preferentially from exogenous AA and this is not inhibited by STZ. These data suggest some interaction between platelets and PMNs in the metabolism of AA.

(iii) the effect of PMNs on platelets does not increase with increasing PMN number (over 1.2×10^7) although PMN AA metabolites, co-chromatographing with PGs, actually decrease with increasing PMN number. This decrease is inversely related to the increase in more polar PMN AA metabolites (likely phospholipids) found with increasing PMN number.

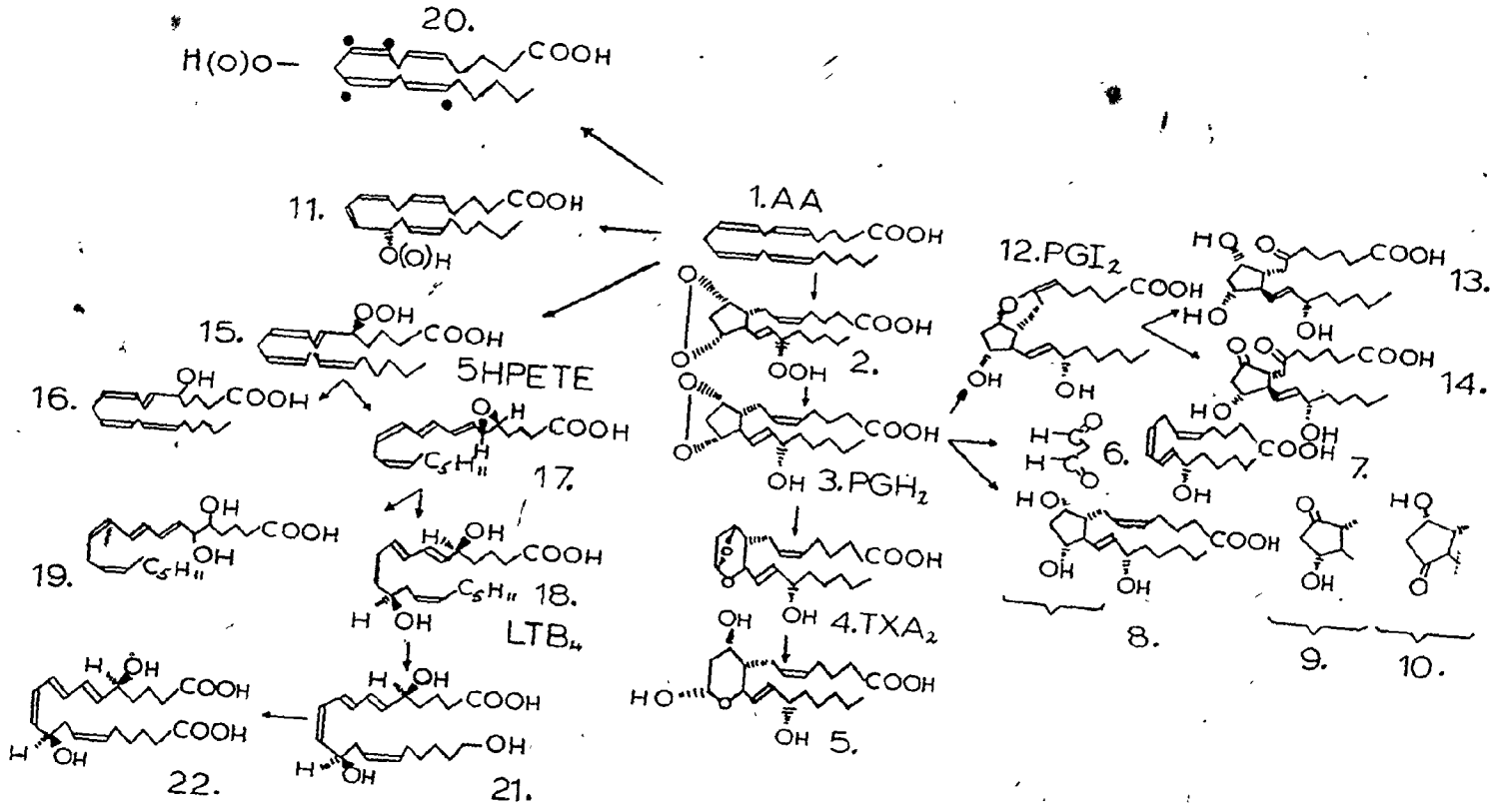
These platelet-PMN interactions in the metabolism of AA may be of importance in the <u>in vivo</u> interactions of these cells as reported in the literature (3.4.1.4).

3.3.1.4 **Discussion**

At the time this work was begun, there were reports that PMNs from a variety of sources produced a variety of cyclo-oxygenase products (3.3.1.1). Borgeat et al (1976) reported on the production of a novel lipoxygenase product by these cells. Since that time a new family of lipoxygenase products and derivatives of lipoxygenase products has been discovered and a new family of biologically active AA metabolites has been classified as "Leukotrienes" by Samuelsson and coworkers (Fig. 37). In a series of papers, Borgeat and Samuelsson (1979a,b,c,d) reported on the production of 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and the corresponding hydroxy acid (5-HETE), 5,12-dihydroxy-eicosatetraenoic acid (5,12-diHETE) and 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) and the corresponding hydroxy acid (15-HETE). The 5,12-diHETE was found to exist in isomers with the 5(S)12(R) isomer being formed enzymatically from an unstable intermediate 5(6)-oxido,7,9,11,14-eicosatetraenoic acid. This intermediate was named Leukotriene A_4 (LTA_4) and the 5(S)12(R)-diHETE isomer was named Leukotriene B_4 (LTB_4). It was discovered that 5-HPETE was the precursor of LTA_4 and hence all products formed from LTA_4 were lipoxygenase metabolites of AA (Fig. 37). Murphy et al (1979) reported that murine mastocytoma cells produced Leukotriene C_4 (LTC_4), a derivative of LTA_4 with a glutathione moiety attached at the 6 carbon position. This and other cysteine derivatives of LTA_4 (LTD_4, LTE_4) were reported to be components of the Slow Reacting Substance (SRS) of Feldberg and Kellaway (1938). PMNs were also found to produce LTC_4 in vitro upon incubation with glutathione (Palmblad et al 1981). Other lipoxygenase derivatives found by these workers are

Fig. 37 The major cyclo-oxygenase and lipoxygenase products of AA metabolism by PMNs, platelets and blood vessels.

1. Arachidonic acid (AA)
2. Prostaglandin G_2 (PGG_2)
3. PGH_2
4. Thromboxane A_2 (TXA_2)
5. TXB_2
6. Malondialdehyde (MDA)
7. 12-hydroxyheptadecatrienoic acid (HHT)
8. $PGF_{2\alpha}$
9. PGE_2
10. PGD_2
11. 12-hydroxy (hydroperoxy) eicosatetraenoic acid (12-H(P)ETE)
12. Prostacyclin (PGI_2)
13. 6-keto-$PGF_{1\alpha}$
14. 6-keto-PGE_1
15. 5-hydroperoxyeicosatetraenoic acid (5-HPETE)
16. 5-hydroxyeicosatetraenoic acid (5-HETE)
17. 5(6)oxidoeicosatetraenoic acid or Leukotriene A_4 (LTA_4)
18. 5(S)12(R)-dihydroxyeicosatetraenoic acid 5(S)12(R)-diHETE or Leukotriene B_4 (LTB_4)
19. 5,6-dihydroxyeicosatetraenoic acid (5,6-diHETE)
20. 8,9,11 or 15-hydroxy (hydroperoxy) eicosatetraenoic acid (8,9,11 or 15-H(P)ETE)
21. 5,12,20-trihydroxyeicosatetraenoic acid (20-OH-LTB_4)
22. 20-carboxy,5,12-dihydroxyeicosatetraenoic acid (20-COOH-LTB_4)


20.
H(O)O–
COOH
11.
O(O)H
1.AA
2.
OOH
3.PGH2
4.TXA2
5.
12.PGI2
13.
14.
6.
7.
8.
9.
10.
15.
OOH
5HPETE
16.
17.
18.
LTB4
19.
21.
22.

discussed in light of their biological activity in 3.4.1.1. Goetzl and Sun (1979) reported that human PMNs produced a wide range of monohydroxy products of AA including 5-,8-,9-,11-,12- and 15-HETE. Narumiya et al (1981) reported more new metabolites of AA formed by incubation of AA with the partially purified 15 lipoxygenase of PMNs.

The importance of PGs in inflammation has been reviewed (3.1, 3.3.1.1). With the discovery of the leukotrienes, the significance of PG production by PMNs was further clouded. Stenson and Parker (1979) (3.3.1), Smith et al (1981) and Walsh et al (1981) reported that ionophore-stimulated PMNs produced negligible amounts of cyclo-oxygenase products. Bonzer et al (1981a) reported that some HHT production was found. Some of the controversy may be explained in terms of platelet contamination of PMN suspensions (Stenson and Parker 1979). Removal of platelet contamination is difficult and most methods of purification of PMNs from peripheral blood result in drastic loss of cell number in order to achieve reasonable PMN separation from platelets.

Preliminary studies demonstrated that simple purification of PMNs by the method of Ferrante and Thong (1978) led to pure cell populations in terms of separation of PMNs from other leukocytes, however large numbers of platelets remained. These platelets contributed to the AA metabolites recovered especially since the extraction procedure was set up to measure cyclo-oxygenase products. By repeating the cell separation procedure twice (3,3.1.2), substantially less contaminated PMN populations were obtained. This modified technique retained the rapidity and simplicity characteristics of the original technique, cells were not

adversely affected and large numbers of cells were available for study. Using this procedure, platelets were shown no longer to contribute to the AA metabolites measured. Two other facts supported the lack of platelet involvement:

i) the lack of STZ stimulation of platelet AA metabolism. STZ stimulation of PMNs is mediated through C3b receptors on the PMN surface (3.3.1.1).

ii) cells from a donor who had ingested ASA 33 hours earlier provided the same profile of products as PMNs here.

The lack of sensitivity of AA metabolism to indomethacin or ASA inhibition (Fig. 30A) provides strong evidence that the recovered products were not cyclo-oxygenase in origin. This appeared true despite the fact that the products extracted, eluted from silicic acid columns and separated by TLC exactly as PGD_2 and TXB_2. Jakschik et al (1980) used adrenaline, reduced glutathione and combinations of the two to promote cyclo-oxygenase activity and reduce lipoxygenase activity in order to characterize AA metabolites. In our studies adrenaline reduced all products and in no situation were the products increased, suggesting once again, lipoxygenase activity (Fig. 30B). The complete reduction of metabolite formation by NDGA at concentrations that did not block platelet PG synthesis backs up this suggestion. This concentration of NDGA is reportedly specific for lipoxygenase (Morris et al 1980) and blocks HETE production by PMNs (Goetzl 1980).

Intact cells were required to fully produce these STZ-stimulated PMN AA metabolites. In freeze thawed PMNs a peak of products occurred with PGD_2, however this was markedly reduced from intact cells (Fig. 31A). PGD_2 is produced by various blood cells including the platelet.

Steinhoff et al (1980) reported that PGD_2 was produced enzymatically by mast cells and rat basophilic leukemia cells. PGD_2 is produced by other sources (3.3.1.1) and is an important mediator of inflammation (Lewis and Austen 1981). Although ASA and indomethacin did not block the synthesis of this product, it was blocked by 3HMP - a potent PG synthesis inhibitor with unknown lipoxygenase activity. Attempts to separate this AA metabolite from the PGD_2 standard were performed by silica gel TLC using various solvent systems (Fig. 32). Although no separation occurred here, peaks were separated using "Reverse Phase" TLC plates.

Based on this evidence it was concluded that the purer PMN cell population did not produce the regular cyclo-oxygenase products. Siegel et al (1979a,b,c, 1980, 1981) reported that rat pleural PMNs, consisting mainly of stimulated PMNs but also lymphocytes and macrophages, produced HHT, 11-HETE and 15-HETE and upon further stimulation with ionophore A23187, also produced 5-HETE and LTB_4. They reported that ASA blocked 11- and 15-HETE production and also the conversion of 12-HPETE to 12-HETE in platelets. Despite the lack of effect of ASA, the other benzoic acid analogs, some of which inhibit rat paw inflammation, were tested in this system. All "chain" structures, including 2-PBA, 3-PBA, ABA and ASA, had no effect. As reported 3HMP was a potent inhibitor at 250uM. 3-MP caused a shift in the profile of products and an actual increase in products co-chromatographing with PGD_2. This is of interest in light of the report by Claesson et al (1981) that PGI_2 and two PDE inhibitors R020-1724 and 3-isobutyl-1-methylxanthine inhibit the production of LTB_4. The significance of this result is discussed further in 3.4.1.4.

The AA analog 5,8,11,14-eicosatetraynoic acid (ETYA), an inhibitor of both the cyclo-oxygenase and lipoxygenase pathways, also blocked the total profile of products. Initial reports by Borgeat et al (1976) suggested that this agent did not block PMN 5-lipoxygenase. Since that time Goetzl (1980b), Siegel et al (1981), Smith et al (1981) and Walker and Parish (1981) have reported the contrary. Smolen and Weissmann (1980), Marone and Condovelli (1981) and Hafstrom et al (1981) have reported that ETYA blocked the release of lysosomal enzymes from PMNs in response to various stimuli. Bokoch and Reed (1981) reported that ETYA did not decrease but actually increased 5-HETE production by blocking the conversion of 5-HPETE to LTB_4.

Fig. 34 demonstrates that, apart from the more polar products which remain at the origin during TLC using a PG specific solvent system, the majority of AA metabolites move the same distance or further than indomethacin. This indicates that they are less polar products. Jakschik et al (1980) reported that indomethacin co-chromatographed with 5,12-diHETE using this solvent system and Claesson et al (1981) reported that STZ caused PMN 5,12-diHETE production. The peaks at PGD_2 and TXB_2 therefore represent more polar, less widely studied metabolites. These products could be 5,15-diHETE as reported by Doig and Ford-Hutchison (1980) or, more likely, the 20-hydroxy and 20-carboxy ω-oxidation metabolites of LTB_4 (Hansson et al 1981). All monohydroxy compounds are less polar than LTB_4 and would therefore migrate above indomethacin on TLC.

Large amounts of AA were converted to very polar metabolites which were eluted from silicic acid columns with 100% methanol. TLC of this fraction using phospholipid solvent systems revealed peaks of products

migrating with PE and, to a lesser extent PC. This demonstrated that STZ stimulated incorporation of AA into membrane phospholipids. Stenson and Parker (1979) reported a high degree of incorporation of 5-HETE into membrane fractions, triglycerides mainly in nonstimulated cells and phospholipid in A23187 stimulated cells. 5-HETE was rapidly esterified into cellular lipids whereas other lipoxygenase products were not. These authors were the first to report the incorporation of exogenous 5-HETE into membranes. Walsh et al (1981) reported that 30% of HETE produced by PMNs was incorporated into cell membranes. Bonser et al (1981a) reported that A23187 released AA from PE and PC stores in the membranes of DMSO differentiated human promyelocytic leukemia cells.

In our studies STZ stimulated the incorporation of AA or AA metabolites into cell membranes of PMNs but not platelets (Fig. 29B,C). Indomethacin, at high concentrations, caused a slight reduction in incorporation of AA (Fig. 30A). The AA incorporation required intact cells (Fig. 31A) and was blocked by ETYA and 3HMP but not NDGA. This suggests a different mechanism of action of these agents, which all blocked the 6% MeOH/$CHCl_3$ products. Fig. 34 demonstrates the large percent of exogenous AA incorporated in phospholipid - that peak of radioactivity which remains at the origin. This incorporation may be related to phagocytosis as FMLP increased 6% MeOH/$CHCl_3$ products without affecting the 100% methanol fraction.

Stenson and Parker (1979) reported that although platelet contamination was present in their PMN cell suspensions, PMNs prelabelled with AA produced PGE_2. To investigate the possibility that AA had to be incorporated into specific phospholipids in order to be available as substrate for the cyclo-oxygenase, cells were prelabelled

with AA and then incubated unstimulated. Fig. 35 shows that PG products were negligible compared to other products both more and less polar. ASA appeared to cause minor, nonPG related changes.

The possibility of platelet involvement in reports of PMN PG synthesis was further evaluated. Small numbers of platelets were found to preferentially produce PGE_2 under the present conditions. This was a function of platelet number (Fig. 29B,30D,36A,B). STZ did not increase but decreased the amount of PGE_2 produced. In PMN cell suspensions, platelets did not respond in a similar manner. These cells produced TXB_2 as the major peak - a peak which increased upon addition of STZ. This activity was sensitive to ASA. These studies point to a role of platelets in the reported PG production by PMNs and, more important, they point to an interaction between platelets and PMNs in the metabolism of AA. This has not been reported to date. Reports do exist concerning the interaction of platelets and PMNs in adherence to vessel walls (Stewart et al 1977, Ratcliff et al 1979), production of procoagulant activity (Niemetz et al 1977) and platelet satellitism (McGregor et al 1980) (a phenomenon where platelets surround and are eventually engulfed by PMNs). Ratcliff et al (1979) found PMNs covering denuded canine arteries, however this adherence was intimately mediated through platelets. Also local inflammatory processes may be associated with intravascular activation of PMNs (Cunningham et al 1980). Leukoembolism, with platelet involvement, may be involved in pulmonary embolism (Jacob et al 1980) and leukocytes may help mural thrombi attach to vessel walls (Stewart et al 1977). Finally PMNs produce platelet activating factor (PAF)(Lotner et al 1980 2.3.1.1). Some or all of these interactions may have an AA metabolite component. The size of the

TXB_2 peak did not depend on PMN number (Fig. 36B). This would argue against PMNs as a source of this metabolite. The product corresponding to PGD_2 decreased in size as the PMN number and amount of product incorporated into membranes increased. This may indicate an AA product which is either directly or indirectly incorporated into PMN cell membranes.

Platelet - PMN interaction may have importance in vessel injury and intravascular events in inflammation.

In conclusion, these studies of AA metabolism by human peripheral PMNs have shown:

1. Cyclo-oxygenase products from STZ stimulated PMNs are minor, if produced at all, under all conditions tested here.
2. Stimulation of PMNs by STZ leads to increased conversion of AA to lipoxygenase products which co-chromatograph, under the usual TLC conditions, with standard PGs.
3. Of the ASA-like drugs tested, 3-MP caused a shift in profile of 6% $MeOH/CHCl_3$ products to the less polar product while 3HMP eliminated all products in this silicic acid column fraction. ASA and other NSAID had no effect.
4. NDGA and ETYA, known lipoxygenase inhibitors, blocked production of metabolites co-chromatographing with the PG standards.
5. Reverse TLC may be a simple, effective way to separate these products from the regular PGs.
6. The products that co-chromatograph with the PGs are more polar than LTB_4 and hence, possibly the ω-hydroxy and carboxy oxidation products of LTB_4.
7. Stimulated PMNs incorporated large amounts of AA or AA metabolites

into membrane phospholipids.

8. ETYA, as well as 3HMP, blocked the incorporation of AA into phospholipid although NDGA did not, suggesting different mechanisms of action. 3-MP also had no effect.

9. Small numbers of platelets (less than 5×10^7/ml) produce PGE_2 in larger quantities than TXB_2 and this may explain reports of PGE_2 production by PMNs which are not free of platelets.

10. Platelets, in the presence of PMNs, preferentially produce TXB_2 suggesting an interaction between these cells, especially in the presence of STZ.

11. This method for separation of PMNs retains all the qualities of the original method described by Ferrante and Thong (1978) but reduces platelet contamination to negligible amounts.

3.4 In Vivo Effects of Benzoic Acid Analogs on Inflammation

3.4.1 Effects of Benzoic Acid Analogs on the Rat Pleurisy Model

3.4.1.1 Introduction

The role of PGs as possible mediators of inflammation has been discussed (3.1, 3.3.1.1, Vane 1976). Some of the most convincing evidence for a role for PGs in inflammation, apart from their biological activity, is the correlation between PG synthesis inhibitory effects and anti-inflammatory activity of NSAID. The discovery of a new family of AA metabolites (3.3.1.4) has given further evidence of more complex involvement of AA in inflammation (Higgs et al 1979). The biological activities of the newly discovered lipoxygenase products are diverse and some effects of these compounds will be summarized here.

The leukocyte chemotactic activity of the platelet derived lipoxygenase product of AA metabolism, 12-HETE, was first reported by Turner et al (1975). 5-HETE has been reported to have chemotactic and chemokinetic activity (increases cell random movement) (Goetzl et al 1981), to cause plasma exudation (Higgs et al 1981) and to release lysozyme from the specific granules of human neutrophils (Stenson and Parker 1980) although this latter effect has been questioned (Häfstrom et al 1981). Like most chemotactic substances, 5-HETE, when incubated with PMNs, induces a refractoriness in the cells that is specific to chemotactic stimulation by 5-HETE. The hydroperoxy precursor of 5-HETE, 5-HPETE, in contrast, induces nonspecific unresponsiveness to all

chemotactic agents, possibly through covalent binding or metabolite formation (Goetzl et al 1981). Other HETE and HPETEs cause chemotactic activity, induce neutrophil aggregation, enhance expression of neutrophil complement (C3b) receptors (which promote phagocytosis) and elevate intracellular cGMP levels (Goetzl et al 1980).

After 5-HETE, LTB_4 is the second major AA lipoxygenase product produced by PMNs. Three isomers of LTB_4 exist (Ford-Hutchison et al 1981), all of which have biological activity. The 5(S)12(R) isomer is produced enzymatically and is the most potent. LTB_4 has been found in the synovial fluid of patients with rheumatoid arthritis and other forms of arthritis (Klickstein et al 1980). LTB_4 causes chemokinesis (Carr et al 1980, Ford-Hutchison et al 1981, Palmblad et al 1981), aggregation of rat PMNs (Ford-Hutchison et al 1981), changes in blood vessel permeability in conjunction with PGE_2 (Ford-Hutchison et al 1981, Higgs et al 1981), increases in blood flow to some extent (Bray et al 1981a), lysosomal enzyme release (Hafstrom et al 1981, Palmblad et al 1981, Rae and Smith 1981) and neutropenia (Bray et al 1981b) as well as other effects in the immune response.

Doig and Ford-Hutchison (1981) reported a product of AA lipoxygenase activity that was more polar than LTB_4 and that had PMN aggregating and chemotactic effects.

15-HPETE inhibits PMN leukotriene synthesis (Vanderhoek et al 1980).

On guinea pig lung parenchymal strips, LTC_4, 20-OH-LTB_4, LTB_4, 20-COOH-LTB_4 (Fig. 37) and histamine caused contraction, in that order of potency. (Hansson et al 1981). LTB_4 was the most potent of the metabolites to cause leukocyte adhesion and chemotaxis. LTC_4 and D_4 were

reported to have similar activity to angiotensin in causing intense arteriolar constriction of short duration (Dahlen et al 1981) and this was followed by extravasation of macromolecules from post capillary venules. Although these compounds were much more potent than histamine, LTB_4 did not cause this effect.

The effects of various drugs provide further evidence for a role of AA lipoxygenase products in inflammation. Indomethacin and ETYA have been reported to inhibit release of PMN lysosomal enzymes induced by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP), although ASA was ineffective even at very high doses (Smolen and Weissmann 1980). Further, AA-induced enzyme release was blocked by the cyclo-oxygenase and lipoxygenase inhibitor ETYA in a dose-dependent fashion (Hafstrom et al 1981). NDGA, at doses specific for the lipoxygenase enzyme activity, depleted PMNs of HETEs and inhibited random and chemotactic migration and this could be reversed by the addition of HETEs (Goetzl et al 1980, Goetzl 1980b). Concerning the mechanism of action of HETEs, Stenson and Parker (1979) were the first to report that the incorporation of 5-HETE or other lipoxygenase products into PMN membranes was an important metabolic pathway of these compounds and that this may mediate the phagocytic and chemotactic activities of these cells. Siegel et al (1980) suggested that salicylates inhibited inflammation by inhibiting the enzyme 12-HPETE peroxidase, which converts 12-HPETE to 12-HETE, which would in turn elevate HPETE which would block formation of the inflammatory mediator PGI_2.

Higgs et al (1979) suggested that an interaction between the PG and lipoxygenase products may ultimately control inflammation. Lewis and

Austen (1981) classified interactions between the two systems as follows:

1. antagonism of biosynthesis (PGI_2 inhibits SRS-A release)
2. augmentation of metabolite formation (SRS-A causes TXA_2 release)
3. interaction at a target tissue (LTB_4 plus PGE_2 in inflammation - see below).

Wedmore and Williams (1981) proposed that PMNs interact with the endothelium to supply macromolecules to the site of acute inflammation. In this report, LTB_4, C5a and FMLP were all found to act synergistically with PGE_2 but not histamine or bradykinin, in causing edema formation. The increased vascular permeability caused by these interactions however, was dependent on the presence of PMNs. These agents served as chemical signals to which the PMN responded, by some unknown mechanism and this indicates a central role for PMNs in the inflammatory process.

Vinegar et al (1973) reported on the carrageenin-induced rat pleurisy model as a screen for anti-inflammatory drugs. Subplantar injection of carrageenin had been shown previously to produce edema and inflammation in the subcutaneous tissue of the rat hindlimb while intraperitoneal injection of carrageenin was shown to produce an exudate (Vinegar et al 1973).

Carrageenin is a sulfated polygalactan derived from certain species of red algae and is used as an emulsifier and stabilizer in the food industry. It causes granuloma formation (tumor-like mass of tissue with actively growing fibroblasts and capillary buds) and interacts with the blood coagulation and kinin systems. The lambda fraction, which contains equal portions of 1,3 linked and 1,4 linked D-galactose units in a

double helical conformation with covalent interchain bonds, is most inflammatory and is effective at concentrations well below the toxic levels (Di Rosa 1972, Thompson and Flower 1981). Carrageenin has pronounced effects on blood platelets, leukocytes and blood pressure when administered systemically (Vargaftig and Le Fort 1977).

Although the carrageenin rat paw model of inflammation has been widely used to test anti-inflammatory compounds, the rat pleurisy model has the advantage of allowing precise determination of the cell type and number at the site of inflammation (Vinegar et al 1973). In this model a sharp increase in leukocytes, mainly PMNs, entering the pleural cavity occurred between 1 and 3 hours after the injection of carrageenin. The maximum volume increase occurred by 5 hours. PMN levels reached maximal levels between the 3rd and 5th hour. ASA had only a transient inhibitory effect on exudate volume but completely inhibited further PMN migration after 3 hours. In this model there was a 2 hour delay before monocyte mobilization occurred, but this mobilization increased with time in a linear fashion and, like exudate volume, was also transiently inhibited by ASA. It appeared that edema formation and PMN mobilizaton were related however, ASA completely blocked PMN and monocyte mobilization at times when the effects on exudate volume were waning (Vinegar et al 1973, Almeida et al 1980). Almeida et al (1980) also studied the effect of NSAID in the rat pleurisy model and found that indomethacin was effective when given orally 30 minutes prior to or 90 minutes after intrapleural carrageenin. It appeared more potent in the latter case. The accumulation of neutrophils in this study was dependent on the dose of carrageenin and the size of the animal.

Vinegar et al (1980) reported a biphasic edematous component to

this inflammation model with one phase occurring within the 1-5 hour period after carrageenin and the second phase at 5-9 hours. The neutrophil accumulation pattern was monophasic, occurring within 1-5 hours while monocytes accumulated at 3-7 hours.

Apart from the NSAID, caffeine was shown to potentiate the acute anti-inflammatory activity of ASA but not through enhanced inhibition of PG synthetase (Vinegar et al 1976). Caffeine alone inhibited the edema component and blocked neutrophil mobilization, possibly through a cyclic nucleotide mechanism (Vinegar et al 1976). Caffeine did not potentiate the anti-inflammatory effects of sodium salicylate but only the effect of drugs which worked through a PG synthetase mechanism.

The rat pleurisy model of inflammation has been reviewed by Van Arman (1978). This report also points to the effects of an additional simultaneous site of injury on a site to be studied. For example, administration of some irritant at a site different from the original site of inflammation, causes a dose related inhibition of pain and white cell accumulation at the original site.

Issekutz and Movat (1980) noted maximum neutrophil accumulation 2-3 hours after subcutaneous injection of chemotactic stimuli in rabbits and they suggested that the short time course may be due to the decline in chemotactic substances generated in the lesions or to vascular damage, stasis, hemorrhage and thrombosis that compromise the circulation and delivery of neutrophils to the site.

The carrageenin-induced inflammation models have been widely used to assess the anti-inflammatory activity of chemical agents, of which the mechanisms of anti-inflammatory activity are variable (Vinegar et al 1973, Vinegar et al 1976, Almeida et al 1980). Cerskus and Philp (1981)

studied the effects of the benzoic acid analogs on inflammation using a carrageenin-induced rat paw inflammation model. In this study, the oral administration of 3-MP, ABA and 2-PBA reduced inflammation in doses comparable to ASA. 3-PBA did not supress edema formation. The anti-inflammatory activity could not be explained in terms of PG synthesis inhibition and this work suggested that compounds with chemical structures similar to ASA exhibited anti-inflammatory effects through diverse mechanisms.

In the following study, the effect of these benzoic acid analogs on neutrophil accumulation in vivo was investigated as a possible site of nonPG mediated inhibition of edema formation. This was based on work suggesting a central role of the PMN in acute inflammatory events (Wedmore and Williams 1981).

3.4.1.2 **Methods**

The study of the effects of the benzoic acid derivatives on in vivo neutrophil accumulation was based on the method of Vinegar et al (1973) with modifications as described below.

Male Sprague-Dawley rats, 200-250 g in weight, were anesthetized with urethane as described in the thrombosis study. Animals were randomized in groups of eight and drugs were administered in a single dose through I.V. injection as described in the thrombosis study. Drugs were used at high concentrations found acceptable in the thrombosis study: ASA, ABA, 2-PBA and 3-PBA at 100 mg/kg, 3-MP at 50 mg/kg and 3HMP at 10 mg/kg. The control was 50% PEG in saline which was the vehicle for all drugs and the volume administered was always less than 0.3ml.

Carrageenin (Type Lambda, Sigma Chemical Co., St. Louis) was made up as a 2 mg/ml solution in sterile water and this was injected into the pleural space between the 3rd and 5th ribs, on the right side of the mediastinum, using a 26 gauge, 1/2 inch needle. Carrageenin was administered immediately after drug injection. Animals were kept warm by heat from a light bulb and were sacrificed by chloroform inhalation 4 hours after the administration of carrageenin. The thorax was opened by a lateral incision just above the diaphragm and along both sides of the rib cage. The pleural exudate was collected in a 1ml plastic disposable syringe using a 20 gauge needle and the volume was measured and recorded. The cavity was washed twice with 2.5ml of 0.10% EDTA in saline to give a final volume of 5.0ml plus exudate volume. The total leukocyte cell number was determined as described in the PMN study, by counting 50ul of this suspension. The cells were centrifuged at 200 x g for 10 minutes and the pellet was resuspended in 200ul of human serum which had been previously stored at -20° C. A smear of these cells was made as described in the PMN study and a differential count of PMNs, eosinophils and mononuclear cells was made after staining the smear with Wright's Stain (Appendix III).

The effect of carrageenin administration in this model was compared to sterile water controls and the total leukocyte and PMN cells and the exudate volumes were compared by the student's t-test for unpaired data. Due to heterogeneity of variances between these groups, all statistical analyses in this study were performed on logarithmic transformed data. Drug vehicle was also used in all animals in this study.

The effects of various drug treatments on this model were compared by performing individual analysis of variance (ANOVA) and Tukey's

Honestly Significant Difference Test (Wilcox et al 1979) for each of total leukocyte, PMN, eosinophil and mononuclear cell counts and also exudate volumes.

3.4.1.3 **Results**

Rat pleurisy exudates contain mononuclear leukocytes as well as PMNs (Plate 5). In comparing the carrageenin-induced pleurisy to the sterile water control animals, certain differences were apparent. Although the recoverable exudate volume was low in the carrageenin group, there was a statistically significant increase over the controls ($p < 0.001$) (Table 3). The total leukocyte numbers increased 6 fold in the carrageenin group ($p < 0.001$) and this was mainly due to the large influx of PMNs, which increased from 0.10×10^6 cells to 37.5×10^6 cells per rat pleural cavity (Table 3).

In comparing all drugs to the vehicle control, no agent significantly inhibited pleural exudate volume (Table 4). There was a trend towards inhibition with 2-PBA however. 3-PBA caused a statistically significant increase in exudate volume, with a 2 fold increase, on the average, compared to the controls.

3-MP and 3HMP were the only agents to cause statistically significant reductions in the total leukocyte count and this was due to decreases in PMN infiltration. Although 3-PBA did not significantly reduce total leukocyte number, there was a trend towards reduction and this was reflected in the statistically significant reduction in PMN accumulation. There was a trend to inhibition with 2-PBA but this did not reach statistical significance in either PMN or total leukocyte

Plate 5 Photomicrograph of cells isolated from the rat pleurisy model.

Cells were removed from the rat pleural cavity 4 hours after the administration of 500 ug carrageenin, and resuspended in human blood serum.
A typical blood-type smear of this cell suspension was done and stained with Wright's stain to visualize the cells.
In addition to PMNs, other cells, including lymphocytes, can be found in this preparation.
x 1000.

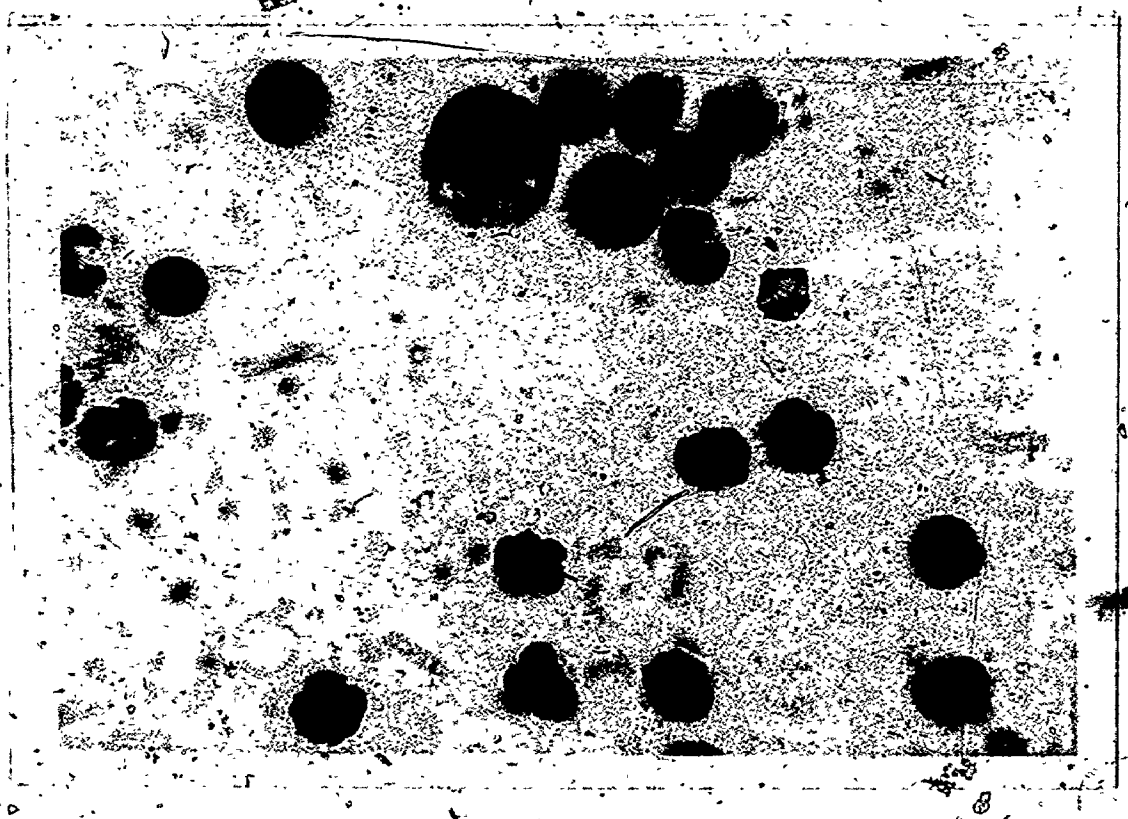


TABLE 3 THE RAT PLEURISY MODEL: EFFECTS OF INTRAPLEURAL CARRAGEENIN ON PLEURAL EXUDATE VOLUME AND LEUKOCYTE ACCUMULATION

Conditions	n	Exudate Volume (ml)	Total Cells ($x10^6$)	PMNs ($x10^6$)
Sterile Water (Control)	8	0.09 ± 0.01	7.05 ± 0.74	0.10 ± 0.03
Sterile Water + Carrageenin	8	0.28 ± 0.06	43.46 ± 5.46	37.54 ± 4.78
		p<.001*	p<.001	p<.001

* compared to controls by student's t-test for unpaired data

TABLE 4 EFFECTS OF BENZOIC ACID ANALOGS ON THE RAT PLEURISY MODEL (mean ± SEM)

Parameter	n	Control	ASA 100mg/kg	2-PBA 100mg/kg	3-PBA 100mg/kg	ABA 100mg/kg	3-MP 50mg/kg	3HMP 10mg/kg
Exudate Volume (ml)	8	0.28 ± 0.06	0.24 ± 0.03	0.15 ± 0.04	0.58 ± 0.15*	0.31 ± 0.04	0.21 ± 0.05	0.21 ± 0.02
Total WBC ($x10^6$)	8	43.46 ± 5.46	35.26 ± 2.78	26.55 ± 4.81	26.53 ± 5.43	30.14 ± 4.22	13.85 ± 2.13*	19.44 ± 5.49*
PMNs ($x10^6$)	8	37.54 ± 4.78	30.75 ± 2.79	19.60 ± 5.06	18.64 ± 5.76*	24.84 ± 4.55	7.38 ± 2.35*	14.10 ± 5.37*
Eosinophils ($x10^6$)	8	1.04 ± 0.37	0.68 ± 0.07	0.76 ± 0.26	1.07 ± 0.19	0.81 ± 0.23	0.80 ± 0.20	0.67 ± 0.30
Monocytes + Lymphocytes ($x10^6$)	8	4.88 ± 0.89	4.33 ± 0.60	5.71 ± 1.09	6.78 ± 0.64	5.06 ± 0.38	5.66 ± 0.94	4.62 ± 0.74

* $p<0.05$ using Tukey's Honestly Significant Difference test with vehicle control

count.

The only other notable difference was an apparent increase in the influx of mononuclear cells with 3-PBA.

In general, ASA and ABA had no effect on the parameters measured in this model and 2-PBA had no statistically significant effects. 3-MP, at the dose used here, came closest to completely antagonizing the effect of carrageenin. 3HMP was used at a much lower dose than 3-MP and still had potent effects in this model.

3.4.1.4 **Discussion**

Cerskus and Philp (1981) reported on the effects of this series of benzoic acid analogs on the carrageenin-induced rat paw inflammation study. This model allows a quantitative study of the degree of edema formation in response to carrageenin injection. In this study ASA, ABA, 3-MP and 2-PBA caused dose-dependent inhibition of exudate formation and, as 3-MP and ABA were weak or ineffective in inhibiting PG synthesis in rat and human platelets, this demonstrated a dissociation between PG synthesis inhibition and anti-inflammatory activity. Recent evidence (3.4.1.1) indicates that more than PG synthesis inhibition may lead to anti-inflammatory activity and the intimate involvement of PMNs and lipoxygenase products allow possible explanations for these results based on new information.

To evaluate the in vivo effects of these agents on the carrageenin-induced accumulation of PMNs, a modified version of the rat pleurisy model of Vinegar et al (1973) was used. The use of urethane (ethyl carbamate) to anesthetize the animals led to changes in control values compared to those reported for nonanesthetized animals as the

exudate volume was substantially reduced from 0.80 ml/4 hours to 0.28 ml/4 hours, and the PMN accumulation was reduced from 80 x 10^6/4 hours to 40 x 10^6/4 hours. However, in the modified model, carrageenin caused a pronounced increase in exudate volume and PMN accumulation compared to noncarrageenin controls and these model modifications had several advantages for this study. First, the agents could be easily administered I.V., thus reducing variations in absorption kinetics and, to some degree, drug metabolism and also allowing for ready comparisons to effects in the thrombosis model. Secondly, differences in animal activity due to drug effects were eliminated as a variable in the final results.

ASA clearly had no effect on the accumulation of PMNs in this study, in contrast to Vinegar et al (1973) and Almeida et al (1980) who have demonstrated partial inhibition of exudate formation and PMN accumulation. Possible explanations for this lack of effect may be that the urethane has some pharmacologically related effect which masked the effect of ASA or, the route of drug administration may have affected the results. Almeida et al (1980) found indomethacin to be more effective when given 90 minutes after carrageenin administration than 30 minutes before. By using an I.V. route of administration, the ASA may be significantly metabolized before it can exert an anti-inflammatory effect. These factors must also be considered with ABA. Although 2-PBA did not cause any statistically significant effects, there were distinct trends towards inhibition of exudate volume and PMN accumulation. The kinetics of elimination of 2-PBA are not known, however a longer half life would explain the effects of this agent which had similar in vitro effects to ASA on platelet PG and PGI_2 biosynthesis.

3-MP was perhaps the most potent inhibitor of edema formation in the rat paw model (Cerskus and Philp 1981). The weak *in vitro* PG inhibitory effects of this agent suggest some other mechanism of anti-inflammatory activity. The effect of 3-MP on platelet PDE activity (2.3.3.3) provides two new explanations for its effect and its potency. Based on the inhibition of PGI_2 activity by 3-MP (2.4.1.3) and on the report of Hopkins and Gorman (1981) that PDE inhibition in endothelial cells leads to decreased PGI_2 production, which accumulates in this model (Higgs and Salmon 1979), 3-MP may partially block edema formation through indirect PG inhibition. This was not evident in this study however, where the control exudate volume was low. More important however, was the 3-MP mediated decrease in PMN accumulation. This may account for the lack of edema in the carrageenin rat paw model (Cerskus and Philp 1981) if edema is mediated through PMNs as suggested by Wedmore and Williams (1981). Vinegar *et al* (1976) reported that caffeine had anti-inflammatory effects and inhibited PMN accumulation. Elevations in cAMP not only inhibits lysosomal release by these cells but adhesion to vessel walls as well (Trang 1980) and this provides a ready explanation for the lack of PMN accumulation and the subsequent lack of edema formation in the carrageenin rat paw assay.

Although Vinegar *et al* (1973) reported differential effects of ASA on PMNs and exudate formation and Higgs *et al* (1979) reported that NSAID could be given at doses low enough to block PG synthesis and fluid extravasation without inhibiting leukocyte accumulation, the relationship of PMN accumulation to exudate formation is somewhat controversial (Van Arman 1978). In this modified model, there was a decrease in both PMNs and exudate compared to reported controls,

however, the exudate volume appeared inhibited to a larger degree. The effects of 3-PBA provide further evidence for a separation of effects. 3-PBA caused a statistically significant reduction of PMN accumulation and yet caused an increase in exudate formation. 3-PBA has been reported to increase platelet aggregation (Mills et al 1974), ATP release (2.3.1.3), PG synthesis (Cerskus 1978) and PGI_2 activity (2.4.1.3) and it appears to potentiate inflammation at some doses (Cerskus and Philp 1981). An increase in PG production is a possible explanation for the increased exudate formation. If 3-PBA exerts this increased exudate activity through increased PGI_2 synthesis, this would also explain the inhibition of PMN accumulation as PGI_2 inhibits PMN adhesion to vessels (3.1).

All this evidence confirms the hypothesis of Wedmore and Williams (1981) that PMNs, lipoxygenase products and PGs interact in the formation of edema. The effect of 3HMP adds further intrigue to this puzzle of events. 3HMP has not been tested in a carrageenin rat paw model of inflammation. In the rat pleurisy model however, a low dose of 3HMP caused a decrease in the accumulation of PMNs. In vitro, this agent did inhibit the low affinity PDE activity of platelets but it was a very weak inhibitor of PDE in general compared to 3-MP. Goetzl et al (1981) reported that, in contrast to 5-HETE, 5-HPETE, also a peroxide, induced a nonspecific inhibition of PMN chemotaxis to a wide variety of chemotactic agents. These workers proposed that the effect was mediated through covalent binding or formation of an intermediate through the chemically reactive peroxide moiety and this is a possible mechanism of inhibition of PMNs by 3HMP. Work on human PMNs revealed that 3HMP blocked apparent lipoxygenase activity (3.3.1.3). Along these lines 3-MP

caused a shift in products produced. Stenson and Parker (1979) suggested that the incorporation of lipoxygenase products into PMN membranes was associated with cell activity and inhibition of lipoxygenase enzyme activity blocked this cell activity. The potent effects of this agent on PG synthesis must also be considered. Ex vivo studies with rat blood indicates that although this agent is a potent PG synthesis inhibitor in vitro, the effects are less pronounced ex vivo. The lipoxygenase inhibition component of the effects of 3HMP appears, at present, to be the most likely mechanism of inhibition of PMN accumulation.

These results, in light of current work on acute inflammation, provide certain answers to the diversity of effects of the benzoic acid analogs on rat paw inflammation. The importance of PMN involvement is evident by the effects of 3-MP. Further confirmation of this point would result if 3HMP could be shown to have anti-inflammatory activity, however the contribution of the antiPG effects of this agent must also be considered.

The dissociation of PMN accumulation and exudate volume has been demonstrated by:

(i) the levels of PMNs and exudate volume found in this model compared to reported controls and

(ii) the results of 3-PBA.

Small numbers of PMNs may still potentiate exudate formation despite this evidence dissociating PMN accumulation from exudate formation. The anti-inflammatory effect of ASA, ABA and 2-PBA as found in the rat paw model are not readily explained by the results in the rat pleurisy model. Although inhibition of PG synthesis is a likely explanation for the effects of ASA and 2-PBA, the mechanism of action of

ABA remains unknown and further investigation may lead to a new understanding of inflammation, especially in light of the antithrombotic activity of this agent in the rat thrombosis model.

From these data it is necessary to use the hypotheses of PMN involvement, lipoxygenase inhibition and PG synthesis inhibition in order to explain the diversity of effects of these benzoic acid analogs on inflammation.

Chapter 4 GENERAL DISCUSSION AND CONCLUSIONS

This work does not represent the classical structure-activity study aimed at establishing an optimal molecular configuration for a desired pharmacological effect, but it represents the novel approach of using structural analogs of an important pharmacological agent, namely ASA, as tools for gaining a better understanding of some of the pathological processes modified by ASA, specifically thrombosis and inflammation. The purpose of this project was to add to a base of information about a series of structural analogs of ASA and their effects in thrombosis and inflammation in order to characterize the properties of ASA which confer these effects and to differentiate, if possible, the sites of action of each activity. The overlap of antithrombotic and anti-inflammatory activities has hampered the use of ASA as an anti-inflammatory agent and the antithrombotic action of ASA requires clearer definition. This work has been directed at the PG related effects of these agents, however it has been necessary to consider other sites of action to explain certain biological effects not related to PG synthesis inhibition.

As ASA is the prototype agent upon which this study was based, the biological effects of other agents will be compared to those of ASA. New information and conclusions drawn from this work will be underlined and Table 5 is a general summary of the present knowledge concerning these agents.

In studies of platelet function, ASA had no effect on the first phase of ADP-induced platelet aggregation but it blocked second phase aggregation and ATP release at a lower concentration than any of the

TABLE 5 SUMMARY OF EFFECTS OF BENZOIC ACID ANALOGS IN THROMBOSIS & INFLAMMATION

Benzoic Acids	Platelet							Blood Vessel		PMN	Models			
	Agg.[a,1,2] 1st	2nd	ATP[b]	PG[c]	PDE[d] cA I	cA II	cG	PGI_2 Activity	AA Metabolism	AA Metabolism	Rat[3] Thrombosis	Rat[e] Pleurisy PMN Acc	Exu	Rat Paw[2] Inflammation
ASA	-	++++	++++	++++	-	-	-	+++	++++	-	++++	-	-	+++
2PBA	-	+++	+++	+++	-	-	-	+++	+++	-	+++	-	-	++++
3PBA	-	P	P	-	-	-	-	P	-	-	+	++	P	P
ABA	-+	-+	-	-	-	-	-	-+	-+	-	+++	-	-	+++
3MP	+++	+++	+++	-+	+++	+++	+++	+++	-	++	+	++++	-	+++
3HMP	++++	++++	++++	++++	+	-	-	++++	P	++++	-	++++	-	NT

\+ inhibition - no effect

P potentiation NT not tested

[a] 1st, 2nd phase aggregation

[b] ATP release

[c] PG synthesis inhibition

[d] PDE activity: cAMP I (low affinity), cAMP II (high affinity), cGMP

[e] Rat Pleurisy: PMN accumulation, Exudate volume

see also:

1. Mills *etal* (1974)
2. Cerskus and Philp (1981)
3. Philp *etal* (1978)

other agents and irreversibly inhibited platelet PG synthesis. ASA did not inhibit platelet PDE activity. In the blood vessel, ASA inhibited PGI_2-like biological activity in a concentration related way and blocked blood vessel mediated conversion of AA to products which co-chromatographed with PG standards such as PGI_2 and E_2. ASA blocked thrombus formation, in the rat model described here, but it cannot be concluded that this occurred solely through a PG-related mechanism as ASA did not block thrombus formation at a dose that blocked rat platelet PG synthesis ex vivo. Only extremely high doses were effective on the regular 15 minute post drug injury although lower doses were effective in preventing thrombus formation with a 10 minute post drug injury. This phenomenon is unexplained. It is generally accepted that ASA blocks the second phase of platelet aggregation by inhibiting platelet PG synthesis and that ASA blocks PGI_2 synthesis by blood vessels. The antithrombotic effect of ASA has been related to its inhibition of platelet PG synthesis. The data reported here indicate that a high dose of ASA blocks electrically-induced thrombus formation by some mechanism other than irreversible PG synthesis inhibition. ASA actually increased the tendency to thrombus formation at some concentrations in this model and this could be related to the inhibition of PGI_2 production.

PMNs can enzymatically oxidize AA to products which co-chromatograph with standard PGs in a process not blocked by ASA and this may be important in inflammation.

ASA has been shown to block carrageenin-induced rat paw edema when given orally, however ASA, administered intravenously, did not block PMN accumulation in the rat pleurisy model used here. Inhibition of PMN accumulation is not a major mechanism by which ASA exhibits

anti-inflammatory activity and PG synthesis is not necessary for PMN accumulation.

2-PBA had the identical in vitro spectrum of activity to ASA but at a lower potency. Therefore, extension of the acetoxy chain of ASA by 1 carbon unit to propionyloxy allows retension of the effects of ASA on second phase aggregation, ATP release, platelet PG synthesis, PGI_2-like activity inhibition, AA metabolism by aorta tissue and anti-inflammatory activity. This agent caused the same effects as seen with ASA in the tests made here, but it was less well tolerated by the animals at high I.V. doses (200 mg/kg). Because of this similarity to ASA, 2-PBA may prove useful in the study of the irreversible inhibition of PG synthesis by ASA and for studying the different sensitivities of various PG synthetases to inhibition by ASA.

Despite the in vitro results indicating that ASA was a more potent PG synthesis inhibitor, 2-PBA was reported to inhibit rat paw edema at a lower concentration than ASA and in tests for the inhibition of PMN accumulation, the effect of 2-PBA was of borderline significance while that of ASA was not. The anti-inflammatory effects of this agent in relation to ASA require further investigation especially in view of pharmacokinetic properties, before assigning a PG inhibitory mechanism to the anti-inflammatory effect of ASA.

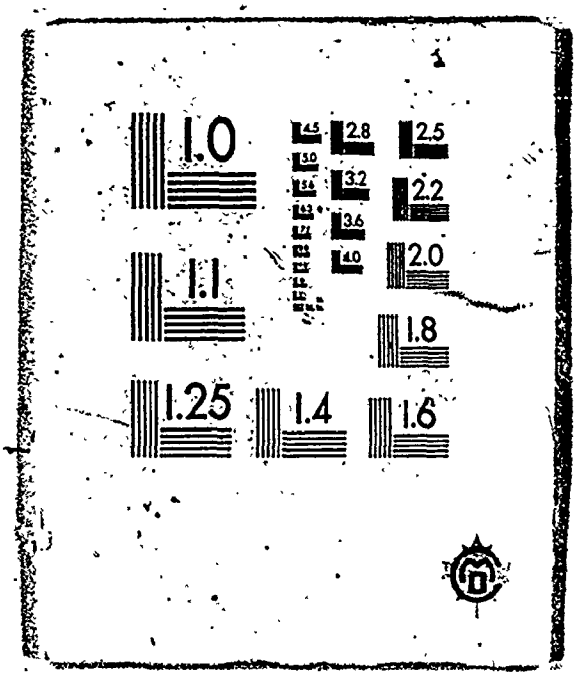
3-PBA was of particular interest initially because of the tendency towards increased, instead of decreased, ADP-induced platelet aggregation by platelets incubated in its presence. It was also shown to increase the production of proaggregatory substances from platelet

microsomes incubated with AA, however this effect was not reflected in increased conversion of AA to PGs in studies of platelet PG synthesis. Despite the lack of effects on the profile of platelet AA metabolites and on the conversion of AA to metabolites by rat and rabbit aorta tissue, 3-PBA caused increased ADP-stimulated platelet ATP release at some concentrations and an increase in PGI_2-like activity produced by rabbit aorta. Both of these effects were small in magnitude. 3-PBA was the only agent to alter exudate formation in the rat pleurisy model and, in this model, 3-PBA significantly increased exudate formation. Based on the platelet and PGI_2 data, this is likely through a PG-mediated mechanism. The statistically significant reduction in PMN accumulation also shown here, associated with the increased exudate formation, suggest that PGs (including PGI_2) play a role in limiting the accumulation of PMNs at inflammatory sites.

3-PBA displayed a weak trend towards the inhibition of thrombus formation and the mechanism could be one of two speculative possibilities. 3-PBA may inhibit thrombus formation by increasing PGI_2 formation or, alternatively, it may work by some common, as yet unknown, mechanism as suggested for ASA.

In general, this agent is a useful tool for understanding the role of PGs in inflammation and for investigating alternative mechanisms of antithrombotic activity.

ABA is closely related to ASA chemically in that it lacks only the ester oxygen, but this difference allows the molecule to exist in two tautomeric forms. Furthermore, the small change in chemical structure results in a complete loss of PG inhibitory effect in platelets and the



low dose inhibitory effect on second phase platelet aggregation as seen with ASA. ABA had no effect on the profile of platelet AA metabolites and caused a low, variable, nonconcentration related inhibition of PGI_2-like activity. ABA did inhibit thrombus formation by an unexplained mechanism. The effect of ABA was not dose dependent and unlike ASA, caused a more consistent inhibition of thrombus formation at 100 mg/kg than at 200 mg/kg. This provides further evidence that the antithrombotic activity seen in this model by these agents are not mediated solely by PG synthesis inhibition. The claim has been made that ABA has anti-inflammatory activity, not related to inhibition of PG synthesis. ABA did not block PMN conversion of AA to more polar products nor did it reduce the accumulation of PMNs in the rat pleurisy model. An understanding of the anti-inflammatory action of ABA may uncover a new component to the inflammatory process. Similarly, an understanding of the antithrombotic activity of ABA may lead to a better understanding of thrombus formation. If this agent proves to be as useful an anti-inflammatory agent as ASA, without blocking normal platelet function, this would be a significant therapeutic advance.

3-MP was originally prepared in this laboratory to resemble the ring tautomer of ABA. In contrast to ABA, this agent was a much more potent inhibitor of both phases of ADP-induced platelet aggregation and release of ATP. 3-MP was a weak inhibitor of platelet PG synthesis and did not alter the profile of platelet derived AA metabolites. 3-MP did block both the high and low affinity cAMP PDE activity and cGMP PDE activity and this provides a plausible explanation for the inhibition of both phases of platelet function as inhibition of cAMP PDE would lead to

elevations of intracellular cAMP. 3-MP blocked the production of PGI_2-like activity despite weak antiplatelet PG effects. In this regard, 3-MP was only a weak inhibitor of AA metabolism by the blood vessel, therefore, PGI_2-like activity is likely inhibited by an indirect mechanism such as inhibition of aorta cell PDE. 3-MP had only weak antithrombotic effects despite its potent effect on platelets. The PDE inhibition by an ASA analog however, hints at a direct relationship between PGs and cyclic nucleotides.

3-MP was reported to have potent anti-inflammatory effects in the rat paw carrageenin model. 3-MP blocked the accumulation of PMNs in the rat pleurisy model used here and, based on the importance of PMNs in edema formation, this is likely the mechanism of 3-MP anti-inflammatory activity. The mechanism of PMN inhibition may be through PMN PDE inhibition, elevation in cAMP and the subsequent inhibition of cell function or through some alteration in lipoxygenase product formation. These effects have been demonstrated in vivo. These phenomena may also be related. The indirect inhibition of PGI_2 activity and its role in blocking inflammation is important and must also be investigated. For an ASA-like agent, 3-MP demonstrates novel activities and like ABA, may represent another alternative approach to anti-inflammatory activity which is mediated through PG synthesis inhibition.

In the study of fatty acids that are enzymatically oxidized to potent biological molecules, to find a molecule such as 3-MP which

(i) is a structural analog of a potent irreversible PG synthesis inhibitor but is itself a very weak inhibitor and

(ii) itself spontaneously photo-oxidizes to a potent inhibitor of

cyclo-oxygenase and other enzymatic oxidation processes (3HMP), is an oddly coincidental discovery. 3HMP was initially shown to block both phases of ADP-induced platelet aggregation and was more potent than ASA in inhibiting platelet PG synthesis. The mechanism of action of PG inhibition is unclear but may be related to other peroxide inhibitors of PG synthesis. 3HMP blocked ADP-induced platelet release of ATP and was the most potent inhibitor of PGI_2-like activity from aorta rings. 3HMP caused a massive increase in AA oxidation products in the presence of aorta tissue and although this may occur through a nonspecific chemical interaction, there was a tissue requirement and hence the mechanism is not known. In contrast to 3-MP, 3HMP had no PDE activity except for a weak effect on the low affinity cAMP PDE activity, hence the mechanism for the biphasic inhibition of platelet aggregation is not known. 3HMP blocked completely the conversion of AA to oxidized metabolites by the PMN. 3HMP blocked the incorporation of AA metabolites into PMN membrane components. 3HMP was a potent inhibitor of PMN accumulation and it is proposed that 3HMP exerts its potent inhibitory effects on PMN accumulation through the inhibition of AA lipoxygenase metabolism and subsequent mediator formation. The anti-inflammatory effects of this agent have yet to be determined especially in light of the effects of this agent on PGs and PMNs. 3HMP was the most potent PG synthesis inhibitor and the effects on PMN accumulation indicate that it is active in vivo despite a lack of effect on rat blood vessels or platelets ex vivo. These findings lead to questions regarding the sensitivity of PG synthetase and lipoxygenase systems to peroxides in vivo. The lack of effect of 3HMP on thrombus formation is understandable in light of the ex vivo effects of this agent, which blocked neither PGI_2-like activity

nor AA-induced rat platelet aggregation after intravenous administration.

ASA and 2-PBA were irreversible PG synthesis inhibitors and were the most potent inhibitors of second phase aggregation and release of ATP showing the importance of PG synthesis to ATP release. 3-MP and 3HMP were inhibitors of both phases of platelet aggregation but were not more potent inhibitors of ATP release. Therefore we were unable to separate drug effects on the release of ATP from effects on second phase platelet aggregation.

In the platelet lysate PG preparation, two peaks of metabolites consisting of the antiaggregatory PGD_2 and the stable end product of the proaggregatory TXA_2, TXB_2, predominate under the conditions used here. Imidazole, a specific thromboxane synthetase inhibitor, inhibited not only the TXB_2 peak but also the PGD_2 peak and led to an elevation of PGE_2. This indicates a relation between TXA_2 and PGD_2 formation. The benzoic acid analogs inhibited platelet PG synthesis in the order of potency previously reported and inhibitors blocked both the PGD_2 and TXA_2 peaks with no concomitant increase in PGE_2, indicating cyclo-oxygenase inhibition.

In no situation was a separation of antiplatelet and antiPGI_2 activity possible with the agents tested. PG synthesis inhibitors inhibited both activities (ASA, 2-PBA and 3HMP), agents without inhibitory effects on platelets had no inhibitory effect or had potentiating effects on both systems (ABA and 3-PBA) and 3-MP blocked

PGI_2-like activity and platelet function apparently through indirect mechanisms.

Cyclo-oxygenase products from STZ stimulated PMNs are minor, if produced at all. None of the standard PGs were detected here. Stimulation of PMNs by STZ however, leads to increased conversion of AA to lipoxygenase products which co-chromatograph, under the usual PG TLC conditions, with the standard PGs. Production was blocked by NDGA and ETYA which are known lipoxygenase and cyclo-oxygenase inhibitors but not by NSAID which are specific PG synthesis inhibitors. The products are more polar than LTB_4 and are possibly ω-oxidation products of LTB_4.

The reverse phase TLC procedure used here may prove useful as a method for studying these agents as this technique allowed separation of these metabolites from the PG standards.

Stimulated PMNs incorporate large amounts of AA or AA metabolites into membrane phospholipids and this is blocked by ETYA and 3HMP but not by NDGA, suggesting different mechanisms of action of lipoxygenase inhibition.

The degree of platelet contamination of PMN samples must be addressed for all PMN preparations, as small numbers of platelets produce significant amounts of PGE_2, which has likely been attributed to PMNs in the past. Furthermore, platelets in the presence of PMNs produce increased amounts of TXB_2, suggesting an interaction between platelets and PMNs in AA metabolism.

The method of Ferrante and Thong (1978) as modified here for the separation of PMN populations reduces platelet contamination to negligible amounts but retains the advantages of speed, low cost and gentle cell treatment in the purification of PMNs.

SUMMARY

1. ASA blocked thrombus formation in the rat model. This required high doses and was not solely related to the regular PG synthesis inhibition. ASA at lower doses actually increased the trend to thrombus formation. The effect of the time of post drug injury on the antithrombotic effect of ASA further supports the claim that the antithrombotic effect of ASA, seen here, is not solely related to irreversible PG synthesis inhibition.

ASA did not inhibit PMN accumulation in the rat pleurisy model used here, suggesting that this PMN accumulation is not dependent on PG synthesis.

2. 2-PBA is a unique agent in the sense that it possessed all the *in vitro* activities of ASA but at reduced potency. This agent could be used in the study of irreversible cyclo-oxygenase inhibition and for studying variable sensitivities to inhibition of cyclo-oxygenase from different tissues.

The apparent increased activity of 2-PBA in the rat paw inflammation model and on PMN accumulation in the rat pleurisy model used here, despite decreased *in vitro* activity, make this agent useful for studying the mechanisms of anti-inflammatory action by ASA.

3. 3-PBA clearly potentiated many PG related biological phenomena now including platelet ATP release and PGI_2-like activity from the vessel wall.

3-PBA significantly increased exudate formation in the rat pleurisy

model and significantly inhibited PMN accumulation. These results, in light of the *in vitro* data, suggest that increased PG$_o$ (including PGI$_2$) synthesis results in increased exudate formation and PGs may, in fact, limit the degree of PMN accumulation. This inverse relationship dissociates exudate formation and PMN accumulation and has not been previously reported.

Trends towards antithrombotic activity with 3-PBA point to either a nonPG related phenomenon or a PGI$_2$ mediated antithrombotic effect.

4. ABA was not a PG synthesis inhibitor but blocked thrombus formation providing further evidence for a nonPG related antithrombotic effect.

ABA has been reported to have anti-inflammatory activity and this was not related to *in vitro* PG synthesis inhibition, PMN accumulation inhibition or PMN AA metabolism inhibition.

If further studies confirm that ABA has anti-inflammatory activity without blocking *ex vivo* platelet function, as indicated by these results, this agent may prove to be an alternative to ASA for treating inflammatory conditions without causing the platelet function defects associated with ASA. This is a significant finding.

If ABA has antithrombotic activity in other models, this would point to new mechanisms of antithrombotic activity perhaps not related to PG production.

ABA is a useful tool for separating PG related and nonrelated phenomena associated with the biological effects of ASA, and ABA may represent a significant therapeutic advance in the treatment of both thrombosis and inflammation.

5. 3-MP has potent inhibitory effects on platelet function, PGI_2-like activity and PMN accumulation. 3-MP was however, only a weak PG synthesis inhibitor. The finding that 3-MP was a potent inhibitor of platelet PDE leads to a possible explanation of the observed phenomena. 3-MP inhibition of PDE could lead to an elevation of cyclic nucleotides which has been shown to mediate inhibition of platelet function, endothelial cell PGI_2 production and PMN activity. The fact that a structural analog of ASA works through this mechanism suggests some direct relation between cyclic nucleotide and PG systems.

3-MP inhibition of PMN accumulation provides an explanation for its anti-inflammatory action and suggests a possible route for further examination of alternate anti-inflammatory mechanisms.

3-MP alters PMN AA metabolism. This may be a direct effect or one related to PDE metabolism.

3-MP is an example of an agent which displays anti-inflammatory activity through alternative mechanisms and further information about its mechanism of action will increase the understanding of inflammation.

6. 3HMP is a hydroperoxy compound shown to have a vast array of <u>in vitro</u> and <u>in vivo</u> activity and the understanding of the actions of this hydroperoxide may lead to as yet unrealized interactions of fatty acid oxidation in biological and pathological events. Such phenomena as complete aggregation inhibition is not usual for an agent working exclusively through PG synthesis inhibition. 3HMP was only weak as a PDE inhibitor ruling out this possible mechanism of action. The role of lipoxygenase products may be important here (see below) or alternatively, 3HMP may have direct membrane effects.

The potentiation of AA oxidation by aorta tissue by 3HMP shown here is important for understanding the role of the blood vessel in AA metabolism. The phenomenon is particularly significant considering that 3HMP was the most potent inhibitor of PGI_2-like activity. The lack of antithrombotic activity and weak ex vivo activity of 3HMP are surprising and important in light of the effects in the rat pleurisy model.

The potent inhibitory effects of 3HMP on PMN AA metabolism is important and this agent may prove useful in understanding the role of peroxides and peroxidation in this type of AA metabolism.

The potent inhibitory effect of 3HMP on PMN accumulation suggest a role for AA lipoxygenase activity in PMN accumulation. The relationship between this effect and the PDE inhibition by 3-MP must be considered. Here we have two structurally similar compounds exerting the same biological effect but apparently through different mechanisms.

This work with 3HMP suggests different in vivo sensitivities of cyclo-oxygenase and lipoxygenase systems.

7. ASA and 2-PBA were irreversible PG synthesis inhibitors and were the most potent inhibitors of second phase aggregation and release of ATP showing the importance of PG synthesis to ATP release. 3-MP and 3HMP were inhibitors of both phases of platelet aggregation but were not potent inhibitors of ATP release. Therefore we were unable to separate drug effects on the release of ATP from effects on second phase of platelet aggregation.

8. In the platelet lysate PG preparation, two peaks of metabolites, consisting of the antiaggregatory PGD_2 and the stable end product of the proaggregatory TXA_2, TXB_2, predominate under the conditions used here. Imidazole, a specific thromboxane synthetase inhibitor, inhibited not only the TXB_2 peak but also the PGD_2 peak and led to an elevation of PGE_2. This indicates a relation between TXA_2 and PGD_2 formation. The benzoic acid analogs inhibited platelet PG synthesis in the order of potency previously reported and inhibitors blocked both the PGD_2 and TXA_2 peaks with no concomitant increase in PGE_2 indicating cyclo-oxygenase inhibition.

9. In no situation was a separation of antiplatelet and antiPGI_2 effects found with the agents tested. PG synthesis inhibitors inhibited both platelet function and PGI_2-like activity production (ASA, 2-PBA and 3HMP), agents without inhibitory effects on platelets had no inhibitory effects or had potentiating effects on PGI_2-like activity (ABA and 3-PBA) and 3-MP blocked PGI_2-like activity and platelet function apparently through indirect mechanisms.

10. Cyclo-oxygenase products from STZ stimulated PMNs are minor, if produced at all. None of the standard PGs were detected here. Stimulation of PMNs by STZ however leads to increased conversion of AA to lipoxygenase products which co-chromatograph, under the usual PG TLC conditions, with the standard PGs. Production was blocked by NDGA and ETYA which are known lipoxygenase and cyclo-oxygenase inhibitors but not by NSAID which are specific PG synthesis inhibitors. The products are more polar than LTB_4 and are possibly ω-oxidation products of LTB_4.

11. The reverse phase TLC procedure used here may prove to be a useful method for studying these PMN products as this technique allowed separation of these metabolites from the PG standards.

12. Stimulated PMNs incorporate large amounts of AA or AA metabolites into membrane phospholipids and this is blocked by ETYA and 3HMP but not by NDGA, suggesting different mechanisms of action of lipoxygenase inhibition.

13. The degree of platelet contamination of PMN samples must be addressed for all PMN preparations as small numbers of platelets produce significant amounts of PGE_2, which has likely been attributed to PMNs in the past. Furthermore, platelets in the presence of PMNs produce increased amounts of TXB_2 suggesting an interaction between platelets and PMNs in AA metabolism.

14. The method of Ferrante and Thong (1978) as modified here for the separation of PMN populations reduces platelet contamination to negligible amounts but retains the advantages of speed, low cost and gentle cell treatment in the purification of PMNs.

APPENDIX I

EFFECTS OF BENZOIC ACID ANALOGS ON THE RAT THROMBOSIS MODEL: MAXIMUM FALL IN TEMPERATURE OF INJURED CAROTID ARTERY

TABLE 6 EFFECTS OF BENZOIC ACID ANALOGS ON THE RAT THROMBOSIS MODEL (15 min post drug injury) II. MAXIMUM FALL IN TEMPERATURE OF INJURED ARTERY

Drug	n	Max. Fall in Temp. of Carotid Artery After Injury (mean ± SEM) Control (°C) Right Artery	Drug (°C) Left Artery
Control 50% PEG/Sal.	6	1.5 ± 0.1	1.6 ± 0.1
ASA 33mg/kg	4	1.3 ± 0.2	1.9 ± 0.4
ASA 10mg/kg	6	1.2 ± 0.1	1.2 ± 0.01
ASA 100mg/kg	6	1.4 ± 0.2	1.6 ± 0.2
ASA 200mg/kg	6	1.0 ± 0.1	0.6 ± 0.2
2-PBA 50mg/kg	6	1.1 ± 0.1	1.0 ± 0.3
2-PBA 100mg/kg	6	1.1 ± 0.1	1.4 ± 0.1 $p<.02$*
3-PBA 100mg/kg	6	1.4 ± 0.1	1.1 ± 0.3
ABA 50mg/kg	6	1.0 ± 0.2	1.0 ± 0.2
ABA 100mg/kg	6	1.3 ± 0.2	1.6 ± 0.1
ABA 200mg/kg	7	1.0 ± 0.1	0.8 ± 0.2
3-MP 100mg/kg	5	1.0 ± 0.1	1.0 ± 0.2
3HMP 10mg/kg	4	1.2 ± 0.1	1.1 ± 0.3
3HMP 25mg/kg	4	0.7 ± 0.1	1.0 ± 0.2

* compared to controls by student's t-test for paired data

TABLE 7 EFFECTS OF ASA ON THE RAT THROMBOSIS MODEL (10 min post drug injury) II. MAXIMUM FALL IN TEMPERATURE OF INJURED CAROTID ARTERY

Max. Fall in Temp. of Carotid Artery After Injury (mean ± SEM)

Drug	n	Control (^{o}C) Right Artery	Drug (^{o}C) Left Artery
ASA 10mg/kg	6	1.2 ± 0.5	1.3 ± 0.1

APPENDIX II

CHEMICAL SYNTHESIS OF BENZOIC ACID ANALOGS

APPENDIX II

Chemical Synthesis

2-PBA

Salicylic acid (4g) was dissolved in 4g propionic anhydride and 200ul concentrated sulfuric acid were added and the reaction mixture was stirred for 30 minutes in a 53-60° C water bath. The resulting clear solution was transferred to 250ml distilled water and, after agitation, clumps of white solid appeared. The precipitate was filtered and recrystallized a minimum of 2 times from 20% benzene in petroleum ether. TLC of this product was done using ether/petroleum ether (1:4 v/v), methanol/acetic acid/ether/benzene (1:18:60:120 v/v) or cyclohexane/chloroform/acetic acid (50:40:10 v/v) all under saturating conditions, and double or triple developments on Eastman Chromogram TLC plates (2.5 x 8.5cm). Best separation of product from starting materials was achieved with the last solvent system. Plates were stained by spraying with 2% ferric chloride stain which marks phenolic acids. 2-PBA usually appeared in 10 hours while salicylic acid stained immediately, both turning reddish-brown. Melting point 92-95° C (Cerskus 1978).

3-PBA

3-PBA was prepared as 2-PBA, but, substituting m-hydroxybenzoic acid for salicylic acid. Flat white crystals were recrystallized from 20% benzene in petroleum. Melting point 85-87.5° C (Cerskus 1978).

3-MP

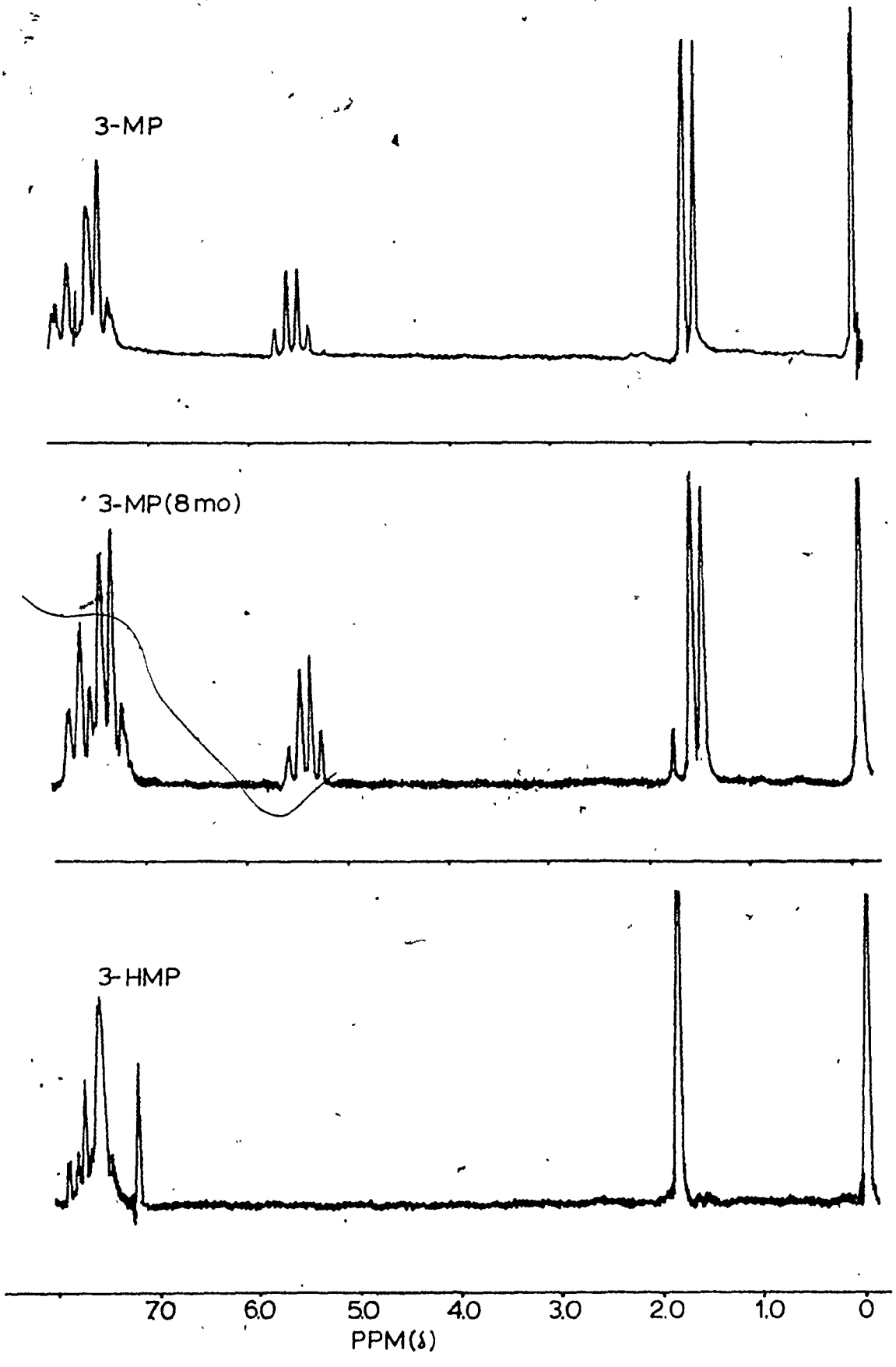
2-acetylbenzoic acid (1.5g) was dissolved in 100ml methanol containing 0.4g sodium hydroxide. Sodium borohydride (0.4g) was added slowly and the mixture was stirred for 18 hours. The reaction mixture was evaporated down to approximately 10ml, 100ml distilled water were added and the mixture was acidified to pH 1 with 6N HCl. The mixture was extracted with chloroform (50ml) 3 times and the pooled chloroform extracts were evaporated to dryness in a rotory evaporator to yield a clear oil with a characteristic "pepper-like" aroma. The oil was distilled in a microdistillation apparatus which did not allow temperature reading. Purity was assessed by nuclear-magnetic resonance (NMR) (Fig. 38) and infrared (IR) spectroscopy, and TLC. The oil was spotted on heat activated Eastman Chromogram TLC plates (2.5 x 8.5cm) and developed 3 times under saturating conditions with 15% ether in petroleum ether. Spots were stained by spraying with anisaldehyde reagent (anisaldehyde/sulfuric acid/95% ethanol 5:5:90) and heated to 100° C for 10 minutes. 3-MP stained white on a brown background. 3-MP was stored at 4° C excluding light.

3HMP

Cerskus (1978) reported that 3-MP underwent photochemical oxidation resulting in a product with potent antiplatelet and PG synthesis inhibitory activity. In our studies, storage of 3-MP in a desiccator exposed to sunlight resulted in the complete conversion to a white precipitate in 365 days. Time course studies on the stability of 3-MP stored at 4° C in the dark with short periods of exposure to light resulted in no formation of detectable oxidation product for 60 days.

Fig. 38 Nuclear Magnetic Resonance (NMR) spectra of 3-MP and 3HMP.

The top spectrum represents newly synthesized 3-MP, the middle spectrum represents 3-MP exposed to sunlight for 8 months and indicates the presence of 3HMP and the bottom spectrum represents purified 3HMP. These spectra are consistent with those reported by Cerskus (1978). The peak at PPM = 0 represents the tetramethylsilane standard.


3-MP
3-MP(8mo)
3-HMP
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
PPM(δ)

The presence of this product, 3HMP, was demonstrated using a sensitive TLC method as described for 3-MP. 3HMP had a smaller R_f value than 3-MP and stained deep purple to red compared to white for 3-MP. 3HMP was separated from 3-MP based on its differential solubility in a 30% hexane in chloroform solution in which 3HMP was precipitated. The presence of 3HMP was confirmed by NMR (Fig. 38). Further tests for 3HMP upon purification included bioassay of inhibitory activity on platelet aggregation and the deactivation of this activity by 10mM ascorbic acid which reduces hydroperoxides. 3HMP lost activity when dissolved in water for 2 hours. Further tests for hydroperoxides were reported by Cerskus (1978).

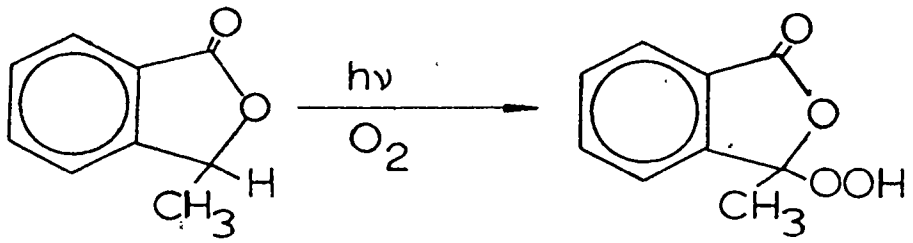
Preliminary studies indicated that newly synthesized 3-MP could be converted to 3HMP in as little as 24 hours by dissolving 3-MP in benzene, bubbling pure oxygen through the mixture and stirring under light from a mercury lamp or short U.V. light source (Fig. 39). All the usual precautions in dealing with highly reactive and explosive peroxide compounds must be observed before scaling up this procedure.

Molecular Weights:

Compound	M.W.
ASA	180.12
2-PBA	194.12
3-PBA	194.12
ABA	164.16
3-MP	148.12
3HMP	180.12

Fig. 39 Reaction scheme for the conversion of 3-MP to 3HMP.

3-MP in the presence of O_2 and light from a mercury or short wave ultraviolet source is converted to substantial amounts of 3HMP in as little as 24 hours.



3-MP 3-HMP

APPENDIX III

REAGENTS, DYES AND BUFFERS

APPENDIX III

Reagents, Dyes and Buffers

Platelet Counting Fluid

A modified Reese-Ecker counting fluid served as both platelet diluant and dye and this consisted of 3.8% trisodium citrate, 0.1% brilliant cresyl blue and 20% formalin from a 40% formaldehyde solution. This dye was filtered each day before use (Levinson and MacFate 1943).

White Cell Counting Fluid

White cell counting fluid consisted of 2% glacial acetic acid and 1% of 1% gentian violet (aqueous) (Levinson and MacFate 1943).

Wright's Stain

Wright's stain dry powder already compounded (Matheson, Coleman and Bell, Cincinatti) (0.1g) was ground for 20-30 minutes in 3-5ml acetone free methanol. Methanol was added to a final volume of 60ml and this was filtered before use (Levinson and MacFate 1943).

Trypan Blue

Trypan blue was prepared by the method of Boyse et al (1964). Trypan blue (BDH, Toronto) was stored as a 0.2% solution in distilled water. Before use 4 parts stock dye were added to 1 part 4.25% NaCl to render it isotonic. PMN viability was assessed by preparing PMNs by the procedure for counting cells but substituting this trypan blue solution for white cell dye. By light microscopy, the total number of PMNs and those taking up trypan blue (indicating cell death) were determined and

the % viability calculated.

Dulbecco's Phosphate Buffered Saline

Dulbecco's phosphate buffered saline was prepared as described by Dulbecco and Vogt (1954) and contained: NaCl 8.0g, KCl 0.2g, Na_2HPO_4 1.15g, KH_2PO_4 0.2g, $CaCl_2$ 0.1g, $MgCl_2 \cdot 6H_2O$ 0.1g in 1L H_2O.

Collagen Suspension

Minced lyophylized shredded bovine Achilles tendon (0.5g) (Grand Island Biochemical Co., New York) was covered with 0.9% NaCl (25ml). The mixture was transferred to the cup of a Sorval Omni Mixer, submerged in ice and then subjected to homogenization at speed 6 for 2 minutes, speed 8 for 2 minutes and speed 9 for 2 minutes. The mixer was stopped intermittently and the strands of collagen cleared from the cutting blades. At the same time portions of 0.9% saline were added to a total volume of 50ml in the mixer cup. Coarse material was removed by centrifugation.

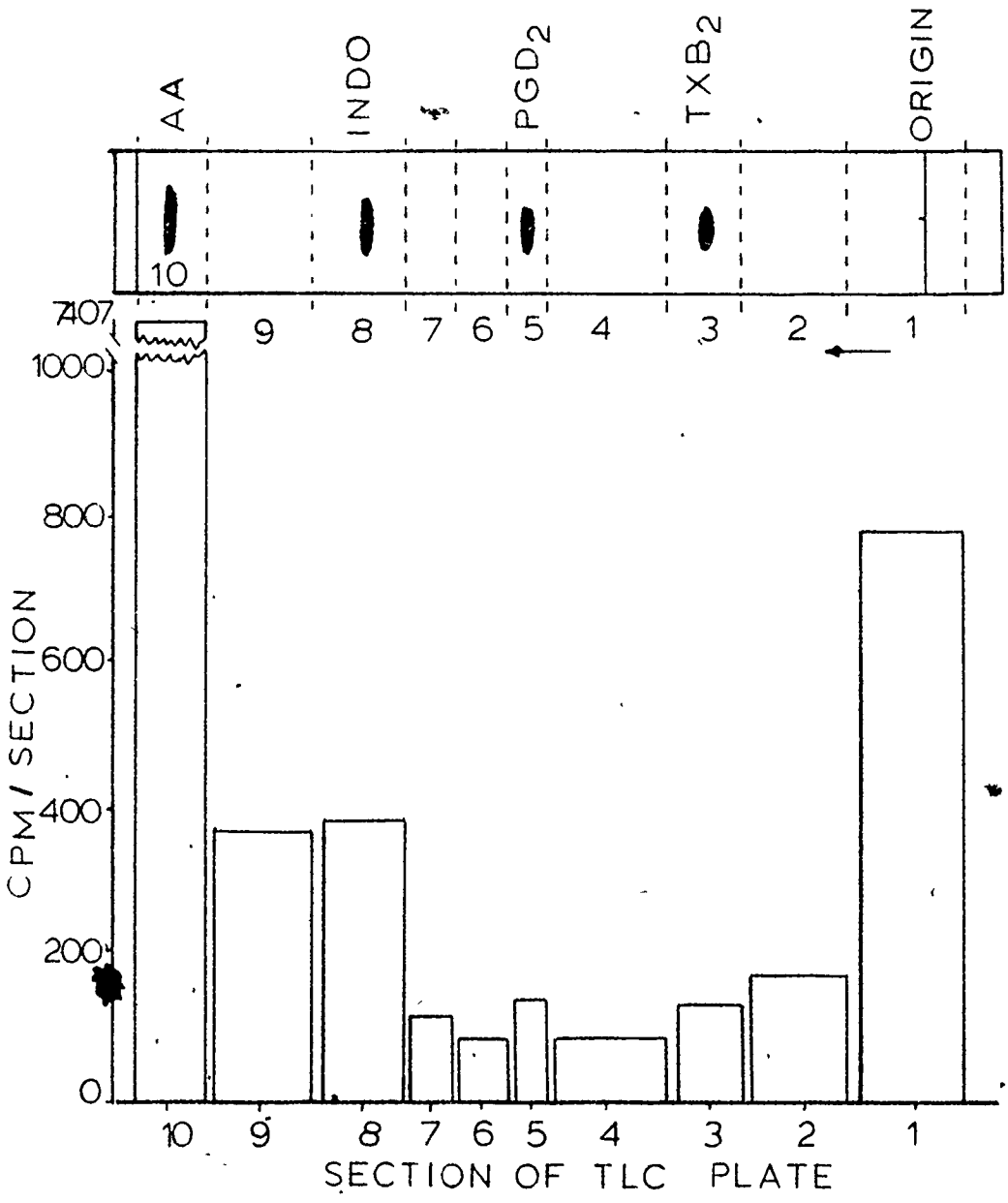
APPENDIX IV

EXPLANATION OF THE GRAPHICAL REPRESENTATION OF THIN LAYER CHROMATOGRAPH DATA

Fig. 40 Typical thin layer chromatograph. Sample and standards were applied to plate at origin and direction of solvent flow is shown by the arrow. Standard compounds were stained, plates were sectioned as indicated by broken lines and each section was counted by liquid scintillation counting. CPM per section are indicated by height of each bar and approximate width of each section is indicated by width of each bar.

AA = arachidonic acid

INDO = indomethacin.


AA
INDO
PGD2
TXB2
ORIGIN
10
9
8
7
6
5
4
3
2
1
1407
1000
800
600
400
200
0
CPM / SECTION
SECTION OF TLC PLATE

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